STRUCTURAL ANALYSES OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM CLINICAL CASES IN VACCINATED AND UNVACCINATED BIRDS

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"STRUCTURAL ANALYSES OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM CLINICAL CASES IN VACCINATED AND UNVACCINATED BIRDS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy
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CERTIFICATE

Certified that this thesis entitled **"STRUCTURAL ANALYSES OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM CLINICAL CASES IN VACCINATED AND UNVACCINATED BIRDS"** is a record of research work done independently by **Shri. Vengadababy, N.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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We, the undersigned members of the Advisory Committee of **Shri. Vengadabady, N.**, a candidate for the degree of Master of Veterinary Science, agree that the thesis entitled **"STRUCTURAL ANALYSES OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM CLINICAL CASES IN VACCINATED AND UNVACCINATED BIRDS"** may be submitted by Shri Vengadabady, N., in partial fulfilment of the requirement for the degree.

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To my beloved Mother

and in loving memory of

my Father

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

INTRODUCTION

Infectious bursal disease (IBD), a contagious viral disease of young chicks, was first reported by Cosgrove (1962) in Gumboro, Delaware, a small village in United States. Since then this disease has been reported from other parts of USA and subsequently from other countries (Okoye, 1984).

Since this virus affect the Bursa of Fabricius (BF) which is concerned with the humoral immune responses of the host, the infected birds are likely to suffer or die from various secondary infections such as gangrenous dermatitis, inclusion body hepatitis, coccidiosis, and colisepticaemia.

The disease usually occurs in two forms, clinical and subclinical, depending on the age at which they get infection and the stage of development of BF. The clinical form is observed in chicks of 5-8 weeks of age with typical lesions of IBD or with highly variable mortality, usually not beyond 20 per cent.

The subclinical form occurs when newly hatched chicks are affected. Though the infection remains undetectable, it causes severe immunosuppression resulting in high susceptibility to secondary bacterial and viral infections, mortality, vaccination failures poor feed conversion, increased condemnation of carcass and medication cost. Of

late, outbreaks of IBD due to a very virulent virus causing dramatic loss upto 80 per cent, not only in chicks but also in pullets in lay, have been reported (Sureshkumar et al., 1984; Chettle et al., 1989). All these lead to heavy economic loss to the poultry farmers.

In India the disease was first reported by Mohanty et al. in 1971 in Uttar Pradesh and subsequently from other states including Kerala (Lalkrishna, 1994). At present the disease is endemic in India and has become the major problem in poultry industry. A mortality of 219,164 birds has been reported between January and October 1993 alone (Lalkrishna, 1994). Vaccination is considered as the only means of controlling this disease. Different types of vaccines in different schedules are being recommended for this purpose (Lasher and Shane, 1994). In areas where antigenic variants have been identified, multivalent vaccines have been suggested. Improved chick quality, good hatchery and farm sanitation practices and effective vaccination programme can reduce the incidence of this disease to a great extent (Lukert, 1994).

In spite of regular vaccination, outbreaks of IBD in vaccinated birds are not uncommon. This lack of protection regularly observed in the field, could be due to a viral mutation resulting from persistence of the virus in the same locality and vaccination pressure leading to increased

virulence, or failure of the field strain to get neutralized by antibodies to the vaccine strain. Hence it is necessary to have a constant surveillance to identify the emergence of new serotypes.

To prevent economic loss from IBD it is important to know whether a flock is sufficiently protected against infection with a given field strain. When protection fails, identification of the pathotype or serotype of the infecting virus and its possible variation from the vaccine strain or other field strain is essential to chalkout vaccination strategies for subsequent flocks.

Identification of different serotypes and subtypes of the virus can be made only with tests that have acceptable degree of specificity and sensitivity. The various tests employed for this purpose are the live bird testing, virus neutralization (VN), ELISA, immunoperoxidase (IP), fluorescent antibody test (FAT) and antigen capture ELISA (AC-ELISA). The live bird test is time consuming, labour intensive and requires high security isolation.

The laboratory tests such as VN, ELISA, IP, FAT and AC-ELISA used with polyclonal antibodies are unsuitable for differentiation of serotypes or subtypes of the virus (Saif, 1994).

Though use of polymerase chain reaction (PCR) generated probe and reverse transcriptase/PCR - restriction enzymes (RT-PCR-RE) are developed to differentiate strains of this virus (Saif, 1994), these tests are costly, requiring highly sophisticated equipments and trained personnel. It is known that protein and nucleic acid analysis of the virus by polyacrylamide gel electrophoresis can identify antigenic variant among the isolates (Jackwood et al., 1984). Though the sensitivity may not be to the extent as that of RT-PCR-RE, these tests are simpler, cheap and can be performed without much difficulty. Taking all the above facts into account, this project was chosen with the following objectives.

1. Isolate IBDV from outbreaks of the disease in vaccinated and unvaccinated flocks.

2. Identify and compare the structural proteins of the field isolates and commercial vaccine strain.

3. Characterize and compare RNAs of the field isolates and commercial vaccine strain.

4. Identify and compare the field isolates and commercial vaccine strain using serological tests namely Agar gel Diffusion test counter immunoelectrophoresis and immunoelectrophoresis.

5. Based on the observations evaluate vaccination break-downs.

Review of Literature

REVIEW OF LITERATURE

Incidence

Infectious bursal disease was first reported by Cosgrove, in young chickens, in the Gumboro area of Southern Delaware, United States (Cosgrove, 1962). Since then such outbreaks were reported from other parts of USA and other countries namely, Australia (Dennett and Bagust, 1980 and Azad et al., 1985), Austria (Vasicek, 1979), Belgium (Devos et al., 1966), Brazil (Nakano et al., 1972), Bulgaria (Lyutskanov et al., 1978), Chad (Provest et al., 1972), China (Zhou et al., 1982), Egypt (Ayoub and Malek, 1976), France (Maire et al., 1969), Germany (Landgref et al., 1967), Ghana (Gyening and Corkish, 1977), Iraq (Mohanty et al., 1981), Israel (Meroz, 1966), Italy (Rinaldi et al., 1965), Japan (Shimizu et al., 1971), Mexico (Giron, 1969), Mauritania (Ba and Chamoiseau, 1977), Netherlands (Lensing, 1968), New Bunswink (Ide and Stevenson, 1973), Senegal (Sayna, 1977), Spain (Badiola et al., 1969), Switzerland (Riggenbach, 1967), Thailand (Ratansethakul, 1983), Yugoslavia (Herceg et al., 1971) and Zambia (Sharma et al., 1977).

In India this disease was first reported by Mohanty et al. (1971) in Uttar Pradesh. Subsequently it was reported from almost all states of the country such as Andhra Pradesh (Jayaramiah and Mallick, 1974; and Sudhakaran et al., 1993),

Bihar (Chauhan et al., 1980), Gujarat (Jhala and Kher, 1991), Haryana (Kumar et al., 1984), Karnataka (Jayaramiah and Mallick, 1975, Aziz and Raghavan, 1986; Aziz, 1988), Madhya Pradesh (Dongaonkar and Rao, 1979), Maharashtra (Dongaonkar and Rao, 1979), Orissa (Rao et al., 1979, Mohanty et al., 1984), Punjab (Anon, 1981 and Anjum, 1994), Tamil Nadu (Jayaramiah and Mallick, 1974; Purushothaman, 1988; Nachimuthu et al., 1993), Tripura (Panisup et al., 1987; Verma et al., 1989) and West Bengal (Bhattacharya et al., 1983; Ray and Sarkar, 1984).

In Kerala the prevalence of this disease was reported by Valsala et al., (1988) and Vijayan et al. (1989) by histopathological examination of bursae from affected birds. Subsequently the virus was isolated from cases of similar outbreaks and confirmed by serological test (Sulochana and Lalithakunjamma, 1991). Since then the disease has been diagnosed in most of the Government and Private farms. Now Kerala seems to be endemic to the disease due to increasing number of poultry farms which provide suitable environment for the virus.

Host affected

Infectious bursal disease is mainly a disease of young chicken, between the age group of 2-7 weeks (Ide and Stevenson, 1973).

Kaufer and Weiss (1980) described that the upper age limit of susceptibility depended upon the age of the chicken at the time of involution of bursa which was the target organ.

Onunkwo (1978) reported that the disease occurred, as early as nine days of age, and the oldest birds infected were 26 weeks of age (Sureshkumar et al., 1984). Though almost all breeds of chicken were susceptible, the severity was high in White Leghorn breeds (Lukert and Witchner, 1984).

Infectious bursal disease was thought to affect only chicken till 1979. In 1979, McNulty and his associates reported the disease in turkey that were naturally suffering from diarrhoea. These authors isolated the virus from faecal samples and detected antibodies in 30 per cent of the serum samples collected from 20-week old turkeys. But Perelman and Heller (1981) observed only an asymptomatic form in turkey as evidenced by neutralizing antibodies in apparently healthy bird.

Louzis et al. (1979) reported a natural outbreak of IBD in pheasant and very recently it was reported in duck (Karunakaran et al., 1992).

Hirose and Hirai (1979) screened egg and serum of quails, geese, bantons and pigeons for the presence of antibody against IBD but failed to detect the same.

Clinical symptoms

The onset of disease was very rapid, indicating a short incubation period of three to four days (Cho and Edgar, 1972).

Depending upon the virulence of the virus, age of chick at the time of infection and maternal antibody status, it may produce either clinical or subclinical disease (Verma et al., 1990).

The initial signs were whitish or watery diarrhoea with straining and soiling of vent feathers and vent pecking. This was followed by anorexia, depression, trembling, severe prostration and death (Cosgrove, 1962, Cho and Edgar, 1972). Mohanty et al. (1971) reported that the affected birds strain for defecation due to vent pecking. Verma et al. (1990) reported significant elevated temperature in the initial stage and subsequent subnormal temperature towards the terminal stage.

In a typical outbreak, 10-20 per cent of the flock showed sudden sign of the disease (Cosgrove, 1962). A higher

morbidity rate upto 60 per cent was reported by Landgraf et al. (1967) and Jayaramiah and Mallick (1975).

The mortality rate in uncomplicated cases was reported to be very low, ranging from 0.5 per cent to 8 per cent (Verma et al., 1990); it was higher when there was secondary bacterial infections such as S. typhimurium (Wyeth, 1975); pseudomonas and E. coli (Bhattacharya et al., 1983).

Chang and Hamilton (1982) and Anjum (1994) observed that aflatoxicosis had a positive effect in making IBD a much more severe disease and changing the symptoms.

Lesions

Cosgrove (1962) described the lesions in the original outbreak, as dehydration, haemorrhages in leg, thigh and breast muscles, hepatic infarction and enlarged kidney. The bursa was enlarged and the ureter was filled with urates. Similar lesions were also observed by Cho and Edgar (1972). Helmboldt and Garner (1964) reported BF as the main target organ showing varying degrees of lesions, depending upon the stage of infection.

Cheville (1967) studied in detail the changes in the bursa, spleen and thymus and found that initially the bursa

had a gelatinus yellowish transudate covering its serosal surface. Later on the longitudinal striation on the surface became prominent and the normal white colour turned to cream and the transudate disappeared as the bursa returned to its normal size and became grey following the period of atrophy.

Haemorrhagic bands were frequently reported on the mucosa, at the junction of proventriculus and gizzard (Verma et al., 1981; Lukert and Hetchner, 1984).

Survanshe (1990) and Anjum (1994) observed pus-like creamy coloured or cheesy plugs in the enlarged bursa and swollen and yellowish reticular appearance of kidney. They have also observed that the gizzard lining was dry and showed one to many erosion areas of variable sizes which could easily slough off.

In addition to the mortality caused by IBDV it also causes immunosuppression which is found to be the major problem for heavy economic loss.

Allan et al. (1972), Faragher (1972) and Rao and Rao (1992) observed the immunosuppressive effect of IBD to Newcastle disease vaccine and they reported that the immunosuppression was greater when chicks below 21 days of age were infected.

Meulmans and Halen (1977) reported low HI titre to Newcastle disease vaccination in IBDV infected birds and high mortality after challenge with virulent NDV.

Lucio and Hitchner (1980) and Winterfield et al. (1980) failed to detect immunosuppression in chicks having maternal antibody and in vaccinated birds.

Panigraghy et al. (1982) observed no effect on secondary immune response to NDV when chicks were infected by IBD on 28 days of age, whereas suppression of primary and secondary immune response to NDV was observed when day old chicks were infected with Poona strain of IBDV (Aziz and Raghavan, 1986).

Giambrone et al. (1977) and Panigraghy et al. (1982) reported suppression of humoral immune response. Singh and Dhawadkar (1994) observed impairment of cell mediated immunity as demonstrated by delayed hyper sensitivity reaction to tuberculin and by total and differential leukocyte counts in IBD affected birds.

The extent of immunosuppression depended on the strain of IBDV involved, age of the birds at the time of exposure to virus, level of maternal antibody, and interval between virus infection and exposure to environmental

pathogen (Faragher et al., 1974; Thornton and Pattisan, 1975; Lucio and Hitchner, 1980).

The immunosuppressed birds failed to produce antibodies in sufficient quantities to counteract the invading pathogens, as evidenced by high mortality of IBD infected chicks with concurrent infections of Newcastle disease (Allan et al. (1972), Salmonellosis and Colibacillosis (Wyeth 1975); Infectious laryngotrancheitis (Rosenberger and Gelb, 1978), Streptococcus fecalis infection (Bhattacharya et al., 1983), Gangrenous dermatitis (Balachandran et al., 1991; Verma et al., 1981); haemorrhagic aplastic anaemia and gangrenous dermatitis (Rosenberger et al., 1975) and aflatoxicosis (Anjum, 1994).

The vaccinal failure as a result of immunosuppression is one of the most deleterious effects of IBD infection, thus nullifying the beneficial effects of the vaccination done to protect the flocks against major diseases, as reported against Newcastle disease vaccination (Ajinkya et al., 1980, Chauhan et al., 1980).

Saif (1991) opined that the reduction in the number of lymphocytes and actively dividing precursors in the BF was probably the most important cause of immunosuppression.

Sharma et al. (1989) observed that the reduction in the number of cells was not only by direct lysis but also by damaging the helper T-cell and by stimulation of suppressor cells.

Etiology

The disease is caused by an RNA virus which is a member of the Birnaviridae family (Dobos et al., 1979), under the genus Birnavirus and the genome of the virus consists of two segments of double stranded RNA, hence the name Birnavirus.

Virus isolation

Chicken embryo

Early attempts for isolation of the virus in chicken embryo was unsuccessful, but it was made possible by Landgraf et al. (1967). They reported a typical experience using the allantoic route of inoculation, but failed to propagate in subsequent passages.

Winterfield (1969) increased the virus concentration in amnio allantoic fluid by serial passage in embryonating eggs. Hitchner (1970) also used the same method to isolate 2512 strain obtained from Winterfield at the 46th embryo

passage and found that the virus concentration reached the peak level at 72 hours post inoculation.

Hitchner (1970) conducted a detailed study for the propagation of IBDV on embryonating eggs. He found that embryos from diseased flock were highly resistant to IBDV and also the allantoic fluid had a very low virus titre than chorioallantoic membrane (CAM). On comparison of different routes it was concluded that the CAM produced high virus titre, followed by yolk sac and least by allantoic route.

Mohanty et al. (1971) observed 5-12 per cent mortality in 10-11 day old embryo whereas Saijo et al. (1990) reported 84 per cent and 90 per cent mortality between 3 and 6 days post inoculation, using two different variants. They also calculated EID 50 in their embryo infectivity assay.

Rao et al. (1978) inoculated three indigenous isolates of IBDV by different routes and found CAM route produced higher percentage of mortality. It was thought that the virus growing in the CAM was less likely to come in contact with the egg yolk which might contain antibodies. They also observed that the rate of mortality increased with the number of passages.

The changes in the infected embryo were dwarfing, oedema, congestion and haemorrhages in the subcutis, greenish

discolouration of liver, necrosis and enlargement of the spleen and pale foci in the heart muscle (Rao et al., 1978)

Based on the above observations CAM is being used for primary isolation of virus by various workers (Hitchner, 1971, Winterfield and Fadly, 1971, Koster and Paulsen, 1971, Amiyakumar et al., 1991, Sulochana and Lalithakunjamma, 1991; Singh and Dhawedkar, 1992).

Mekkes and deWit (1994) opined that though variant strains of IBD could be isolated in eggs by CAM route of inoculation, they generally would not kill the embryos.

Isolation in cell culture

Many strains of IBDV have been adapted to cell cultures of chicken embryo origin.

Landgraf et al. (1967) reported the cytopathic effect (CPE) which included irregular eosinophilic inclusion bodies in paranuclear position in the cytoplasm; and formation of multinucleated cells in chicken kidney cells (Petek and Mandelli, 1968).

Mandelli et al. (1972) and Lukert and Davis (1974) were able to cultivate IBA/PV strain of IBDV in chicken kidney cells and pig kidny cells and they observed CPE in pig kidney cells only at the first passage but upto 15th passage

in chicken kidney cells. Koster and Paulsen (1971) also found CEK cells were highly sensitive to IBDV.

Petek et al. (1973) and Nick et al. (1976) cultivated egg adapted strains of IBDV in chicken embryo fibroblast (CEF) and found that it was most sensitive to the virus isolates than chicken embryo and suckling mice.

Singh and Dhawedkar (1992) could produce cytopathic effects such as clumping of cells and subsequent detachment of cells in chicken embryo fibroblast.

Most laboratories now use CEF for propagation of this virus (Hirai and Shiakura, 1974a; 1974b, Muller et al., 1979; Skeels and Lukert, 1980; Alamsyah et al., 1992).

Lukert and Davis (1974) successfully adapted wild type virus in cells derived from chicken embryo bursa. After four serial passages in chicken embryo bursal cells, the virus could be propagated in chicken kidney cells. They demonstrated plaque production by wild type virus in chicken embryo kidney (CEK) cells. This virus was subsequently propagated in CEF and used as an attenuated live virus vaccine (Skeels and Lukert, 1980).

Dobos (1979) observed that a cell line of Japanese quail origin could support the replication of IBDV.

Yamaguchi et al. (1981) compared susceptibility of lymphoblastoid cells to virulent and attenuated IBDV and found LSCC.BKS and LSCC-CUTO cells were susceptible to both types of IBDV.

Jackwood and Saif (1987) compared three mammalian cell lines (CMA-104, vero and BGM.70) for their ability to support growth of several strains of IBDV serotypes I and II, including serotype I variants and they observed the replication of the viruses in all the cells. They could observe pronounced cytopathic effect in BGM-70 cells, among the three mammalian cells tested.

Infectivity assay in CEF culture was done by Saijo et al. (1990) and they calculated TCID-50. Though the isolation of IBDV from field cases of the disease might be difficult, (McFerren et al., 1980), various workers used cell cultures for virus isolation and for amplification (Jackwood et al., 1985; Jackwood et al., 1989 and Cruz-coy et al., 1993).

Mekkes and deWit (1994) observed that tissue culture could be used for isolation of less pathogenic strains, but virulent strains were difficult to get adapted in this system. Hoveover when passaged in cell cultures virulent strains lost their virulence.

Isolation in chicks

Helmboldt and Garner (1964) experimentally induced IBD in 21 day old chicken, whereas Benton et al. (1967) and Edgar and Cho (1973) produced the disease in 4 week old chicks and Okoye and Uzoukwo (1981) in 4-5 week old chicks.

Mandelli et al. (1969) found that day old chicks were resistant to experimental infection. Wyeth and Cullen (1976) used 8 day old chicks without success but succeeded in infecting 17 day old chicks.

Ide and Stevenson (1973), Wyeth and Cullen (1976), Chauhan et al. (1980) and Mohanty et al. (1981) could isolate the virus from bursal tissue of naturally infected chicks of varying age groups.

Okoye and Uzoukwu (1981) isolated IBDV from the bursal homogenate of four week old chicks infected with IBDV. Lange et al. (1981), Zhou et al. (1982) also reported the susceptibility of 4-5 week old chicks to IBDV.

Though ingestion is the natural route of infection, experimental infection could be produced by different routes.

The disease was produced in two to four days time by intra-occular instillation (Helmboldt and Garner, 1964) intraoccular and intramuscular route (Landgraf et al., 1967; Wood et al., 1981) and crop inoculation (Schneider and Hass, 1967). Intrabursal (Lange et al., 1981) and simultaneous

intracloacal routes (Singh and Dhawadkar, 1994) were also used for experimental infection of chicks.

Ide and Stevenson (1973) could produce only microscopic lesions by SK-1 strain of IBDV in 4 week old chicks, but 100 per cent mortality was observed with strain CU (Kaufer and Weiss, 1980) with peak virus titre within two days after inoculation.

The lesions produced in experimental infection included initial enlargement of the bursa upto the fourth day, followed by regression leading to atrophy by the 23rd day. Hyperaemia of the kidney, thymus and caecal tonsils and depletion of lymphoid cells in the spleen without producing clinical signs and mortality were also observed (Dongaonkar et al. 1979; Verma et al., 1981; Parnisup et al., 1989). However Mohanty et al. (1971) reported 5-12 per cent mortality and 80 per cent morbidity with the same isolate.

Physico-chemical properties

The size of IBDV was described as 10-15 nm in diameter by Benton et al. (1967). Further studies showed variation in diameter 55 nm (Hirai and Shimakura, 1974b) and 58-60 nm (Nick et al., 1976; Jackwood et al., 1982). Nick et al. (1976) also reported the nakedness of the virus.

Hirai and Shimakura (1974b) described an icosahedral configuration in IBDV capsid, consisting of a single layer of 32 capsomeres arranged in 5:3:2 symmetry. The virus was found to have a bouyant density of 1.32 g/ml in cesium

chloride (CSCl) (Nick et al., 1976) and 1.24 g/ml in sodium potassium tartrate. The biochemical studies showed two populations of viral particles with sedimentation coefficient of 395S and 460S.

The virus withstood a temperature of 37°C for 90 minutes and 56°C for 5 hours (Benton et al., 1967). It was stable at 60°C for 30 minutes but not at 70°C or 80°C (Landgraf et al., 1967). Cho and Edgar (1969) reported that the virus was stable at 60°C for 90 minutes and was infectious for 21 days at 25°C and for three years at -20°C.

Benton et al. (1967) reported the sensitivity of IBDV to formalin and Wescodyne (an iodine complex), but not to phenol, thiomersol, staphane or hyamine 2389, ether and chloroform. But Cho and Edgar (1969) reported that phenol was lethal at 1 per cent level. They also reported that the virus was resistant to 20 per cent ether for 18 hours or 20 per cent chloroform for 10 minutes at pH 12, whereas Cho and McDonald (1980) indicated the absolute resistance of virus to chloroform at pH 3.

Serotype classification

IBDV was thought to affect only chicken until 1979. McNulty et al. (1979) reported the presence of second serotype isolated from turkey and it showed only 30 per cent relationship with serotype I, detected by virus **neutralization test.**

Lukert and Hitchner (1984) and Lee and Lukert (1987) suggested the possible existence of a third serotype, but Jackwood and Saif (1987) confirmed the existence of only two serotypes.

Jackwood and Saif (1985) studied the cross antigenicity of serotype I and II and found that neutralizing antibodies to serotype II did not protect against serotype I isolate. It was initially thought that VP_3 of four viral proteins (VP_1, VP_2, VP_3 and VP_4) carried the antigenic determinants of serotype specificity (Fahey et al., 1985). However, later studies by Azad et al. (1987), Becht et al. (1988), and Muller et al. (1992) showed that only VP_2 carried the serotype specific antigens responsible for the induction of neutralizing protective antibodies.

Becht et al. (1988) reported that there were at least two virus neutralizing epitopes on VP_2, one of which was strictly serotype specific. On the other hand, Oppling et al. (1991) demonstrated two independent non-overlapping epitopes on VP_3 by virus neutralization tests using monoclonal antibody.

Ture et al. (1993) studied the antigenic relatedness of six classic and variant strains of serotype I and serotype II IBDV using monoclonal and monospecific antibody to VP_2. They have observed that neutralizing antibody against VP_2 of classic and variant strains of serotype I reacted weakly with VP_2 of serotype II.

Strain variation

McFerren et al. (1980) detected antigenic differences among serotype I IBDVS. According to them several field isolates showed only 30 per cent relatedness with the Bursa-Vac-M vaccine strain.

The existence of antigenic variation within serotype I of IBDV has been supported by the detailed genomic studies carried out by Heine et al. (1991). Kibenge et al. (1990), Lana et al. (1992) and Muller et al. (1992) opined that the antigenic variation might occur on the VP_2 region of the IBDV of different isolates.

Bayliss et al. (1990) studied the relatedness of four IBDV strains by comparing the nucleic acid and proteins. All the four strains were closely related. The greatest difference between the two strains was 0.7 per cent at the nucleotide level and 2.7 per cent at the amino acid level.

Van den berg et al. (1991) compared four vaccine strains (PBG 98, D 78, SAC and Winterfield) for their neutralizing antibody against pathogenic strain and they could observe only 20 per cent protection with Winterfield strain, compared to 100 per cent protection by other strains, suggesting the existance of antigenic variation. They also demonstrated a drift that had occurred between the mild and intermediate vaccine strains and pathogenic strain 849 VB, confirming that IBDV was subject to mutation.

Cruz-Coy et al. (1993) compared different isolates of serotype I IBDV using monoclonal antibody and six subtypes have been detected on the basis of neutralization test.

Nachimuthu (1993) and Singh and Dhawedkar (1993) compared different isolates of IBDV using AGDT, CIE and serum neutralization test, and did not observe any antigenic variation.

Recently Vijayapraveen et al. (1995) compared the field isolates with vaccine virus and detected only six proteins in vaccine virus as against seven in field virus.

The frequent antigenic variations due to mutation were randomly controlled by use of current vaccine strain (Muller et al., 1992).

Molecular biology

Structural proteins

Nick et al. (1976) introduced molecular biology in the detection of IBDV based on the structural proteins. They demonstrated four polypeptides using SDS-PAGE, designated as VP_1, VP_2, VP_3 and VP_4, with molecular weights of 110 KD, 50 KD, 35 KD and 25 KD respectively. Similar pattern was observed by Kibenge et al. (1988) and Muller et al. (1992).

Dobos (1979) resolved four polypeptides with minor changes in molecular weights, 90 KD, 41 KD, 35 KD and 28 KD and an additional protein called VPX (47 KD). This was considered as a contaminant protein as it was absent on further purification by two cycles of sucrose gradient and CSCl gradient centrifugation.

Dobos et al. (1979) also reported the same pattern of IBD viral proteins in their studies on biophysical and biochemical characterisation of five animal viruses with bisegmented double stranded RNA. It was also observed by them that VP_2 and VP_3 were the major proteins, forming about 51 per cent and 40 per cent of the total proteins respectively.

Hirai et al. (1979) reported seven polypeptides P_1-P_7 of which P_3 and P_4 were considered as the major proteins, based on their dense staining and high profile. The remaining five were minor bands. The molecular weights of the proteins were 133 KD, 124 KD, 98 KD, 51 KD, 33 KD, 26 KD and 23 KD, respectively.

Jackwood et al. (1984) opined that the additional protein described by Dobos (1979) was a precursor protein of VP_2 but Kibenge et al. (1988) resolved VP-X into two proteins with molecular weights of 48 KD and 49 KD. They also showed 9 proteins in the infectious IBDV.

From the studies on the structural proteins of six isolates of serotype I and two isolates of serotype II Jackwood et al. (1984) opined that the two IBDV serotypes

could be distinguished by their structural proteins when they are grown in CEF cultures. They observed that serotype II lacked VP_2 (42,000) and the molecular weight of VP_3 of serotype I was 34,000 while that of serotype II was 35,500. A difference in molecular weight of VP_4 of serotype I and II was also observed by them, being 28,500 and 27,000 respectively. In addition the major structural proteins of serotype I were VP_2 (42,000) and VP_3 (34,000), while in serotype II VPX (47,000) was the major protein.

Fahey et al. (1985) reported five polypeptides with molecular weights of 91.5 KD, 41.5 KD, 37.KD, 32 KD and 29 KD from 002/73 strain of Australian origin. Lange et al. (1987) also resolved five peptides, but it differed in molecular weights, 95/90 KD, 49 KD, 40 KD, 33/32 KD and 28 KD.

Azad et al. (1985) obtained six fractions of protein having the mol.wt. of 90 KD, 52 KD, 41 KD, 32 KD, 28 KD and 16 KD.

Seven peptides pattern was also reported by various other workers with minor changes in molecular weights such as 82 KD, 74 KD, 44 KD, 37 KD, 30 KD, 28 KD and 26 KD (Kher 1988); 97 KD, 56 KD, 53 KD, 50 KD, 45 KD, 29 KD and 24 KD (Vijayapraveen et al., 1995).

Among the structural proteins, VP_2 has been identified as the major host protective antigen (Becht et al., 1988; Jagadish et al., 1988; Fahey et al., 1989).

Nucleic acid

Muller et al. (1979) studied the IBDV RNA by sedimentation behaviour and SDS-PAGE and reported that the genome contained two pieces of double stranded RNA with molecular weights 2.2×10^6 and 2.5×10^6.

Dobos et al. (1979b) compared the genome of IBDV with Infectious pancreatic Necrosis virus and Drosophila X virus and reported that the bisegmented double stranded RNA genome was characteristic of the birna virus genome.

Lange et al. (1987) studied the structural properties of wild type of IBDV. They analysed the RNA of particles that got separated at two densities 1.29 g/ml and 1.33 g/ml in cesium chloride. Though the protein content of both the fractions were equal, fraction 2 with high buoyant density had higher RNA content. However SDS-PAGE analysis have shown that both the fractions had bisegmented RNA.

The SDS-PAGE had been used extensively for detection of migration pattern of double stranded RNA. Dobos et al. (1979b); Lange et al. (1987) and Singh and Dhawedkar (1993) used this system for comparison of RNA segments of different isolates of IBDV, while Lee et al. (1992) used Agarose gel electrophoresis.

Nick et al. (1976) reported that the gel containing urea influenced the migration pattern of RNA. The migration was slower in gels with urea, than in gels without urea (Muller et al., 1979b).

Differences in the migration pattern of RNA segments of two serotypes of IBDV was also observed in polyacrylamide gel without urea. The migration rate of heavier RNA segments of serotype I and II were similar (Jackwood et al., 1984), but the lighter segment of serotype II migrated faster than the corresponding segment of serotype I (Jackwood et al, 1984; Becht et al., 1988) indicating that RNA migration pattern might help in the differentiation of IBD viral isolates.

Migration distance and direct length measurements under electron microscope was used by Becht et al. (1988) to compare the RNA segments of serotype I and II. They have observed that RNA segment A of serotype II was smaller than the corresponding genome segment of serotype I. Size difference of genome segment B was only slight, the segment of serotype I being slightly larger than that of serotype II.

Diagnosis

Acute clinical outbreaks of IBD in fully susceptible flocks are easily recognized on the basis of history such as rapid onset, high morbidity, spiking mortality and rapid recovery. However, subclinical form poses a problem on diagnosis and the disease may pass unnoticed. A definite diagnosis of the disease require isolation and identification of the causative agent, and serology (Verma et al., 1990).

Serological tests

Various serological tests have been developed for detection of IBDV and its antibody which include tests like SNT, ELISA, immunofluerosence, passive haemagglutination and precipitation tests.

Serum neutralization test

SNT was in use before the application of ELISA for detection of IBD.

Vielitz and Landgraf (1976) used this test to compare the potency of vaccines and for detection of neutralizing antibody in maternally immune and susceptible chicks.

Weisman and Hitchner (1978) and Skeels et al. (1979) used SNT and compared it with AGDT. They reported SNT more sensitive than AGDT.

Naqi et al. (1980) used virus neutralization test to evaluate commercially available IBD vaccines by comparing virus neutralizing titres in vaccinated, vaccinated and exposed, unvaccinated and unvaccinated and exposed chicks.

Jackwood et al. (1982) applied this test to distinguish between serotypes. The VNT was the only serological test that could detect the different serotypes of IBDV and was still the method of choice to detect the antigenic variation between isolates of the virus.

Enzyme-linked immunosorbent assay (ELISA)

Marquardt et al. (1980) first described an indirect ELISA for measuring antibodies against IBDV. Since then several workers have reported on the use of ELISA in IBD diagnosis.

Mallinson et al. (1985), Briggs et al. (1986) and Snyder et al. (1986) used indirect ELISA and compared the same with serum neutralization test. They found ELISA speedier than SNT, with similar sensitivity.

Solona et al. (1985) reported a computer assisted ELISA which was as sensitive as virus neutralization test for rapid detection of antibody titre. This ELISA was found to be more accurate than the conventional ELISA.

Amiyakumar et al. (1991) described an indirect ELISA which can be read without ELISA reader and based on colour reaction.

Fluorescent antibody technique (FAT)

Cheville (1967) employed FAT for detection of antigen in experimentally infected chicks and he could demonstrate antigen in the bursa as early as 48 hours post infection.

Dash et al. (1991b) standardized FAT for the detection of IBDV antibodies and they detected the same antibody on the 4th day of inoculation.

Passive haemagglutination test

In addition to the above serological tests Lin and Gao (1989) introduced a rapid indirect haemagglutination test for the detection of IBD antibody as a diagnostic method.

Braunius and Wit (1990) used the test for detection of IBD antigen from the bursal homogenate by using erythrocyte sensitized with IgG against IBD. They found that the sensitivity of this test was similar to that of ELISA and greater than immunodiffusion.

Subsequently Dzhavadova et al. (1991) used latex agglutination test for detection of IBD antigen as a diagnostic method found that the test was comparable with those of immunodiffusion and immunoelectrophoresis.

Dobos et al. (1979) also reported the same pattern of IBD viral proteins in their studies on biophysical and biochemical characterization of five animal virus with bisegmented double stranded RNA. It was also observed by them that VP_2 and VP_3 are the major proteins forming about 51 per cent and 40 per cent of the total proteins.

Agar gel immuno diffusion test (AGDT)

The simplest method of agar gel diffusion test has been used extensively. Hirai et al. (1972) described AGDT for detection of antibody against IBDV in chicken sera. The appearance of 1 to 3 precipitation lines in 18 to 24 hours at 37°C could confirm the diagnosis (Verma et al., 1990).

The antigen source used for the test varied between laboratories (Wyeth and Cullen, 1976). Weisman and Hitchner (1978) used bursal suspension in different concentration and found that 50 per cent suspension was optimum, whereas Wood et al. (1979) recommended higher antigen concentration for clarity of precipitation line. The other sources of antigen used were embryo allantoic membrane (Wagner and Koster, 1968), embryo liver and CAM of infected embryo (Jayaramaiah and Mallick, 1974, Ganesan et al., 1990 and Sulochana et al., 1991). Because of its simplicity AGDT was being used extensively by several workers in India, for detection of antigen as well as antibody in poultry flocks, following infection or vaccination and for serological screening. This test was being used for diagnostic purpose in Andhra Pradesh (Jayaramaiah and Mallick, 1975, Verma et al., 1981), Bihar (Chauhan et al., 1980), Gujarat (Jhala and Kher, 1991), Haryana (Kumar et al., 1984), Karnataka (Jayaramaiah and Mallick, 1975, Aziz and Raghavan, 1986), Kerala (Sulochana and Lalithkunjamma, 1979), Tamil Nadu (Koteeswaran, 1987), Uttar Pradesh (Pradhan et al., 1983) and in West Bengal (Ray and Sarkar, 1984).

McFerren et al. (1980) employed AGDT for detection of antigenic relationship between two serotypes of IBDV.

Synder et al. (1992) assessed the antigenicity of IBDV using monoclonal antibody in AGDT. It was also employed for the comparison of local isolates of IBDV with standard strain for antigenic variation (Singh and Dhawedkar, 1993;Vijayapraveen et al., 1995).

Cullen and Wyeth (1975) and Wood et al. (1979) developed a quantitative agar gel precipitation test (QAGPT) to quantify antibody. Initially the antigen was titrated against a known positive serum and the titrated antigen was used to quantify antibody by serially diluting the serum to get a clear precipitation line with maximum dilution. This dilution was taken as the end point (Cullen and Wyeth, 1976).

Marquardt et al. (1980) reported the suitability of AGDT for routine diagnosis after comparison with ELISA. Similarly Phillips (1981) compared AGDT with VNT and found that AGDT had 81.5 per cent sensitivity and 100 per cent specificity thereby suggesting AGDT as the simplest method for diagnosis of IBD.

Counter immuno electrophoresis (CIE)

Further advances in IBD diagnostic techniques were made by Berg (1982) who described a rapid counter immuno electrophoresis method for detecting virus specific precipitating antibodies in chicken sera. The method was sensitive, simple and reproducible and detected precipitation after 45 minutes. The result could be read visually and was comparable with that of the AGDT.

Durojaiye et al. (1985) studied the usefulness of CIE in diagnosing IBD in natural outbreaks and they detected antigen in 82 suspected samples out of 89 in a short period of 30 minutes as compared to 24 to 48 hours with AGDT.

Somvanshi et al. (1985) and Dash et al. (1991b) reported increased sensitivity of CIE as it detected antibody as early as 3 to 5 days after infection.

Counter immuno electrophoresis was used to study the antigenic components of IBDV and to detect antigenic variation between different isolates of IBDV (Kher, 1988 and Vijayapraveen et al., 1995).

Immunoelectrophoresis

Talwar (1983) described immunoelectrophoresis technique which was found to be superior than simple immunodiffusion in demarcating different antigenic fractions. This technique was applied for detecting the antigenic fractions of IBDV (Vijayapraveen et al., 1995) and they detected four precipitation arcs in this virus.

Control

Immunization

Immunization is the principal method used for control of IBD in chicken. Primarily vaccination of breeder flocks to confer immunity to their progeny at least in the early stages and secondly immunization of young chicks for active immune response is being advocated (Lukert and Saif, 1991).

Edgar and Cho (1965) vaccinated chicks with bursal extracts from natural outbreak and reported that losses from the vaccinated flocks had averaged less than 0.7 per cent, but the disease could not be eradicated.

Dorn et al. (1968) used bursal homogenates its filtrate and a homogenate of chick embryo inoculated with bursal suspension as vaccine in chicks at 2-12 days of age. They observed slight mortality for four days but the birds were resistant to subsequent infection and their embryo were also resistant to infection. Edgar and Cho (1973) conducted a trial to detect a suitable age for vaccination for a vaccine produced by Auburn university and found 5th week as suitable for vaccination by oral route.

Live vaccine

Winterfield (1969) used an embryo adapted strain (2512) for vaccination and recorded high titre of virus neutralizing antibody 3 weeks after inoculation when vaccination was done at 4 weeks of age and low antibody titre when vaccination done at 3 days of age. Vielitz and Lardgraf (1976) observed that the immunization was effective, when an embryo adapted vaccine was used in chicks after 8 days of age.

Chettle and Wyeth (1986) detected rapid rise of virus neutralizing antibody to $2^{15.3}$ in the first six weeks which persisted upto 18th week.

Winterfield and Thacker (1979) compared eight strains of IBDV as vaccine and found that each strain varied widely in pathogenicity in terms of bursal damage, morbidity and mortality. They also detected varied antibody levels after administration by different routes.

Giambrone and Eckman (1980) administered an attenuated IBDV in drinking water at 18 days of age which protected the birds from clinical IBD at 4 weeks of age.

Wyeth (1980) observed higher antibody levels in chickens vaccinated by oral route than intramuscular injection and the maternal antibody in their progeny was 100 per cent as against 35 per cent of the latter group.

Naqi et al. (1980) evaluated three commercial vaccines and found that the LKT and BV-M strains protected well against bursal atrophy than those vaccinated with BV strain.

Lukert and Mazarctegos (1985) reported that intermediate strains vary in their virulence and could induce bursal atrophy and immunosuppression in one-day-old and 3 week old SPF chicks.

Koteeswaran (1987) reported mild pathological changes in the bursa produced by a vaccine virus resulting in depletion of B cells.

Killed vaccine

Eventhough the live virus vaccine could induce immune response to a higher level and for long time, rapidly declining antibody titre was reported by Wyeth and Cullen (1978). As a solution for the above problem Wyeth and Cullen (1979) introduced an oil adjuvanted killed virus vaccine to boost and prolong immunity in breeder flocks, though they were not desirable for primary response in young chickens.

This vaccine was most effective in chicken that have been primed with live virus.

At present oil adjuvanted vaccine may contain both standard and variant strains of IBDV.

Though many choices of live and killed vaccines are available, a universal vaccination programme cannot be recommended because of the variable immune status of chicks, different management and operational conditions; but killed vaccines are generally given at 16 to 18 weeks of age.

Immunization failure

In spite of strict vaccination programme, vaccination failures have been reported in most of the flock since 1986. Abdu (1986) and Anjum (1994) which have been attributed to various reasons.

Maternal antibody

Antibody transmitted from the hen via the yolk of the egg could protect chicks against early infection with IBDV and resultant protection from immunosuppressive effect of the virus (Lukert and Saif, 1991), whereas the virus neutralizing maternal antibody (MAB) would interfere with the active immune response of chicks to live IBDV vaccines (Wood et al., 1987) and led to immunization failure (Abdu, 1986).

The proper time for vaccination of maternally immune chicks was calculated based on the level of MAB (Lucio and

Hitchner, 1979) and found that chicks having MAB titre below 1:100 were susceptible to infection.

Hitchner (1971) detected low MAB level at 4 weeks and no antibody at 6th week of age. He reported that MAB could not protect the chicks in later stages of life.

Naqi et al. (1983) detected no acceleration of antibody titre in vaccinated birds having declining MAB.

Immunization failure due to inaction of the vaccine virus by high level of MAB was reported by several workers (Wyelt and Cullen, 1976; Skeels et al., 1979 and Abdu, 1986).

Weisman and Hitchner (1978) described a counter action of intermediate invasive strain of IBDV to induce immune response. Wyeth and Cullen (1976) demonstrated complete susceptibility of maternally immune chicks to IBD vaccination at 27 days of age. They studied the susceptibility of chicks having MAB to IBDV at various stages and observed that the susceptibility of birds started at 2 days of age in two flocks and increased gradually and all birds became susceptible at 4 weeks of age, indicating the gradual decay of MAB. Wyeth and Cullen (1978) reported that hens receiving vaccine at later age could protect their chicks upto 22 days of age, compared to 18 days in chicks hatched out from dam vaccinated at early age.

Wood et al. (1981) studied the interaction of MAB with vaccine at two different ages and observed that MAB

prevented response of vaccination at 1 and 14 days of age, but no interference on 28 days of age.

The problems associated with control of IBD are thought to be due to indiscriminate use of vaccines and vaccination schedule resulting in varying MAB levels in chicks (Lukert, 1994; Kreager, 1994); infection due to very virulent strain of IBDV (duPreev, 1994; Kouzenhoven, 1994); high exposure rate (Rosaks, 1994) and multiple serotypes of the virus (Synder et al., 1988; Lukert, 1994).

Materials and Methods

MATERIALS AND METHODS

History

Clinical manifestation, age group affected, morbidity and mortality rate, management practices, including IBD vaccines and vaccination schedule, were all collected in a proforma (appendix) for each specimen.

Glasswares and reagents

During the course of this study Borosil brand of glasswares, Laxbro plastics and analytical or guaranteed reagent grade of chemicals were only used.

Glassware, syringe and needle, filtration assembly and rubber stoppers were processed following standard methods (Hoskins, 1967) and sterilized either in hot air oven or in autoclave depending upon the material to be sterilized.

Reference virus

Infectious bursal disease (IBD) virus received from Madras Veterinary College, was passaged in four week old seronegative chicks, sacrificed on the third day, the bursa collected, processed and stored in small aliquotes at -50°C until used.

Vaccine-virus

Commercially available IBD vaccine strain (Lukert)

was propagated in four week old chicks and the bursal homogenate was stored in small aliquotes at -50°C until use.

Antiserum

Antiserum against the reference virus was raised in four week old IBD seronegative chicks and stored in small aliquotes at -50°C.

Field isolates

Bursae were collected from birds showing clinical symptoms and post mortem lesions. The samples were collected separately from vaccinated and unvaccinated birds, processed, and stored at -50°C to be used as field isolates.

Virus isolation

Materials

Embryonated eggs

Ten day old embryonated eggs from unvaccinated seronegative flocks were received from the University poultry farm, Mannuthy.

Chicken embryo fibroblast (CEF)

Cell culture was prepared from 10 day old embryonated chicken eggs using standard procedure (Hoskins, 1967).

Chicks

Day old chicks from unvaccinated hens received from the university poultry farm were reared upto 4 weeks, without any vaccination for IBD. Before use, the birds were screened for the presence of antibody to infectious bursal disease virus (IBDV).

Processing of clinical samples

The bursae collected from acute cases of IBD in vaccinated and unvaccinated birds were washed in sterile phosphate buffered saline containing antibiotics (500 IU of Penicillin, 500 ug of Streptomycin and 50 units of Gentamicin per ml). It was homogenized in a Tenbroeck tissue grinder to get a 50 per cent suspension using sterile silica gel. The homogenate was frozen at -50°C and thawed at 37°C thrice, centrifuged at 5000 x g for 30 minutes and the supernatant collected. It was then treated with chloroform to a final concentration of 20 per cent for 10 mts and again centrifuged at 5000 x g for 30 minutes. The clear supernatant was collected and used for antigen detection/virus isolation.

Isolation methods

Isolation in developing chick embryos

Well developed 10 day old embryos were candled and those showing brisk movement were selected. The processed samples were inoculated by the CAM route at a dose rate of 100 ul/embryo using five embryos per specimen. The control eggs were inoculated with 100 ul of sterile PBS. After

sealing the inoculation site, the eggs were incubated at 37°C and candled every 24 hours for any mortality. Embryos that died within 24 hours were discarded. Incubation of the eggs were continued until the death of the embryos or for five days. All the dead embryos and embryos alive even after five days were chilled at 4°C overnight before harvesting.

Isolation in chicken embryo fibroblast

Chicken embryo fibroblast culture was prepared by standard procedure in 60 ml capacity Laxbro plastic bottles. After formation of a complete monolayer, it was washed with PBS and infected with 0.6 ml of embryo passaged virus. The infected monolayer was incubated at 37°C for one hour for adsorption. At the end of the adsorption period the inoculum was poured off, washed gently with PBS, fed with maintenance medium containing 4 per cent foetal calf serum and incubated again at 37°C.

The monolayer was examined under an inverted microscope at 24 hour intervals for development of any cytopathic changes. When the CPE was complete the cells were frozen at -50°C and thawed at 37°C thrice for release of intracellular viral particles. The content of the bottle was transferred to a centrifuge tube, spun at 5000 x g for 20 minutes and the supernatant was collected and used for further passage.

Isolation in chicks

One hundred and eighty day old chicks received from University poultry farm were maintained in the laboratory under controlled conditions for four weeks. At four weeks of age they were divided into six groups of 30 each. The first five groups were infected with the bursal homogenate as shown in Table 1.

The sixth group of 30 birds were kept as uninfected controls. The birds were kept in separate cages to avoid cross contamination and observed for three days for any clinical symptoms. On the third day post infection, all the 30 birds from each group were sacrificed and observed for macroscopic lesions of IBD. The bursae from each group were collected and pooled separately.

Table 1. Experimental infection of four week old chicks

Group	Source of virus	Dose	Clinical symptom	Antigen
1	PKD**	100 ul	Ruffled feather and depression	+
2	EKM**	100 ul	Ruffled feather and depression	+
3	THR*	100 ul	Ruffled feather and depression	+
4	KAN*	100 ul	Ruffled feather and depression	+
5	VAC	100 ul	Nil	+
6	NBS	100 ul	Nil	-

* - Vaccinated flock
** - Unvaccinated flock
VAC - Vaccine strain
NBS - Normal bursal suspension

Virus concentration and purification

The bursal samples from each group were processed separately as described above. The supernatent was centrifuged at 15000x g for 15 minutes at 4°C. The clear fluid was treated with 8 per cent polyethylene glycol (PEG) 6000 overnight with constant stirring to precipitate the virus and pelleted by centrifugation at 10,000 x g for 30 minutes. To get maximum recovery of virus, the supernatant was again treated with PEG and subjected to centrifugation at 10000 x g for 30 mts. The pellet was resuspended in sterile PBS to get 1/10th of the original volume, mixed well and centrifuged at 10,000 x g for 30 minutes to get a clear fluid and the pellet containing PEG was discarded.

The clear supernatant was pooled and dialysed against PBS at 4°C to remove traces of PEG by three changes of PBS. After dialysis the clear fluid was centrifuged at 40,000 rpm in Servo Combi Plus No.80 Rotar for 3 hours at 4°C.

The partially purified virus pellet was dissolved in 300 ul of TNE buffer and centrifuged at 10,000 x g for 15 minutes to remove coarse particles by sedimentation. The clear supernatent was used for protein and nucleic acid analysis. The protein concentration was adjusted to 1 mg/ml.

Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used for characterization of protein fractions of different isolates following the procedure described by Laemmli (1970).

Reagents

Solution A

Acrylamide - 30 g

N.N. Methylene bis acrylamide - 0.8 g

Distilled water - 100 ml

The solution was filtered through Whatman No.1 filter paper and stored at 4°C in an amber coloured bottle.

Solution B (pH 8.8)

Tris base - 12.1 g

Distilled water - 80 ml

pH adjusted to 8.8 with 10 N HCl and was made to 100 ml with distilled water, filtered and stored at 4°C.

Solution C (pH 6.8)

Tris base - 6.06 g

Distilled water - 80 ml

pH adjusted to 6.8 with 10 N HCl. Final volume made to 100 ml, filtered and stored at 4°C.

Sodium Dodecyl sulphate (1%)

SDS - 1 g

Distilled water - 100 ml

Filtered and stored at room temperature.

Ammonium persulphate

Ammonium persulphate - 50 mg

Distilled water - 1 ml

Prepared freshly before use

Electrode buffer (Tris Glycine)

Tris base - 3.03 g

Glycine - 14.4 g

SDS - 1 g

Distilled water - 800 ml

pH adjusted to 8.3 with 10 N HCl and the final volume made to 1 litre with distilled water, filtered and stored at room temperature.

Resolving gel (9%)

Solution A	-	6 ml
Solution B	-	7.5 ml
SDS	-	2 ml
Distilled water	-	4.4 ml
TEMED	-	10 ul
Ammonium persulphate	-	0.1 ml
		20 ml

Stacking gel (5%)

Solution A	-	1.65 ml
Solution C	-	2.5 ml
SDS	-	1 ml
Distilled water	-	4.75 ml
TEMED	-	5 ul
Ammonium persulphate	-	50 ul
		10 ml

Sample buffer

Solution C	-	8.5 ml
SDS	-	0.2 g

Glycerol - 1 ml

2 - Mercapto ethanol - 0.5 ml

Pinch of bromophenol blue

Sample preparation

Five samples, two from vaccinated flock designated 'THR' and 'KAN' two from unvaccinated group designated 'PKD' and 'EKM' and a vaccine strain (VAC) were used for protein characterization. Equal volumes of sample buffer and the sample were mixed and heated in a water bath at 90°C for one minute, cooled and stored at 4°C.

Bovine Serum Albumen (BSA) and Chymotrypsin were similarly treated and used as markers.

Staining solution

Coomassie brilliant blue (R-250) - 200 mg

Methanol - 50 ml

Acetic acid - 10 ml

Distilled water - 40 ml

The stain was prepared and filtered through Whatman No.1 filter paper and stored at room temperature.

Destain I

Methanol - 50 ml

Acetic acid - 10 ml

Distilled water - 40 ml

Destain II

Methanol - 25 ml

Acetic acid - 50 ml

Distilled water - 425 ml

Method

SDS-PAGE was carried out according to Laemmli (1970).

The gels were prepared in 16 x 20 cm glass plates supplied with vertical electrophoresis apparatus with 1 mm thick spacer.

Nine per cent resolving gel was prepared (according to the composition) and poured in between the glass plates. Over this 3 ml of distilled water was added to get uniform surface and allowed to polymerise for 20 minutes.

After polymerization water was removed and 5 per cent stacking gel was added and allowed to polymerise for 20 minutes after keeping the comb. The comb was removed after complete polymerization and each well was loaded with

separate samples. BSA and chymotrypsin were also loaded in the same way as markers.

Electrophoresis was initially done at 100 V till the dye reached the surface of resolving gel and then the voltagechanged to 150 V and was continued till the dye reached the bottom of the gel.

The gel was removed and stained overnight with coomassie blue and then destained with decolourizer I for 3 hours, followed by decolourizer II with frequent changes to complete destaining. After destaining the gel was kept on a transilluminator and photographed.

Interpretation

The position of the bands were traced on a transparency sheet. The molecular weights of marker proteins were plotted on a graph in relation to their migration. From this, molecular weights of viral proteins were calculated.

Nucleic acid analysis

Reagents and buffer

Tris HCl (0.2 M)

Tris base - 2.42 g

Distilled water - 80 ml

pH adjusted to 7.5 with 10 N.HCl and the final volume made to 100 ml.

Phenol

Commercially available crystalline phenol was used after boiling.

Ethanol

Commercially available rectified spirit was distilled twice with acid and alkali and stored in rubber stoppered bottle containing slaked lime.

Ethidium bromide (Stock solution 10 mg/ml)

One gram of ethidium bromide was dissolved in distilled water with constant stirring in magnetic stirrer and the solution was transferred to an amber coloured bottle, wrapped with aluminium foil and stored at room temperature.

Tris borate buffer

Tris	-	16 g
Boric acid	-	4 g
Distilled water	-	2000 ml

Sample buffer

Tris (0.2 M)	-	9 ml
Glycine	-	1 ml

Pinch of bromophenol blue

Nucleic acid extraction

Nucleic acid extraction was done by the procedure described by August (1967).

Five virus samples - two from outbreaks in vaccinated flock, two from unvaccinated flock and one IBD vaccine strain (Lukert) were used for nucleic acid analyses.

Equal quantity of phenol was added to 0.2 ml of the purified virus prepared as described above and mixed well for 30 minutes at room temperature. The mixture was then centrifuged at 15,000 x g for 15 minutes. The top layer was removed carefully and nucleic acid was precipitated by addition of two volumes of 70 per cent cold ethanol and the mixture was kept at -20°C overnight (Wallace, 1987).

The precipitated nucleic acid was pelleted by centrifugation at 10,000 rpm for 20 minutes at low temperature (-8°C). The resultant pellet was dissolved in 100 ul of 0.2 M tris (pH 7.5) and was again precipitated with 70 per cent cold ethanol and this procedure was repeated thrice to remove traces of phenol and the final pellet was dried and stored at -20°C.

Preparation of the sample

The pellet was dissolved in 50 ul of 0.2 M tris HCl. Twenty microlitre of this nucleic acid was mixed with 5 ul of sample buffer and stored at -20°C.

Agarose gel electrophoresis

Four hundred and eighty milligram of agarose (Sigma) was added to 60 ml of tris borate buffer and the slurry was kept in a boiling water bath to melt the agarose. When completed, it was cooled to 50°C.

Glass plate of 16 cm x 10 cm size having comb at cathode end was charged with the agarose gel and allowed to solidify. After solidification the comb was removed and the gel was transferred into a submarine electrophoresis chamber containing tris borate buffer. Each well was then loaded with 20 ul viral nucleic acid extracted from different isolates. Electrophoresis was done at 100 V for 4 hours from cathode to anode.

After electrophoresis the gel was removed from the electrophoresis tank and stained with ethidium bromide 0.5 ug/ml for 30 minutes, followed by destaining in distilled water for 30 minutes. Staining of the gel was done in a dark room. After destaining the gel was visualised under ultraviolet transilluminator for the presence of fluorescence and photographed.

Serological methods

IBD viral antigen/antibody was detected by agar gel diffusion test, counter immunoelectrophoresis and immuno-electrophoresis.

Coating of slides

Clean microscopic slides were dipped in 1 per cent agar in distilled water, dried in air by keeping them horizontally over glass rods and stored in slide boxes. These were used for AGDT, CIE and IE.

Agarose for AGDT

Agarose - 0.8 g

Sodium chloride - 8.0 g

Phenol - one drop

Distilled water to make 100 ml

Staining solution

Amido black 10 B - 0.1 g

Sodium chloride - 8.5 g

Distilled water to make 100 ml

Decolourizer I

Methanol - 120 ml

Acetic acid - 30 ml

Distilled water - 30 ml

Decolourizer II

Absolute alcohol - 140 ml

Acetic acid - 20 ml

Distilled water - 40 ml

Antiserum

Antiserum was raised in four week old chicks by administering 100 ul of 10 per cent bursal suspension by occulonasal route. After two weeks they were bled and serum tested for the presence of antibody. When satisfactory results were obtained the birds were bled completely. The sera separated were stored at -20°C for further use.

Agar gel immunodiffusion test (AGID) (Hirai et al., (1972)

The melted agarose was poured on to glass slides and wells were cut in a circular fashion so as to get one central and six peripheral wells. Each of the peripheral well was charged with different bursal suspension in 30 ul quantity, and the central well was loaded with antiserum raised against field isolate. Known positive and negative bursal specimens were included as controls. After charging, the slides were kept in a humid chamber at room temperature for 48 hours and examined against light for the development of any precipitation line.

The slides were washed by soaking in two changes of normal saline for 24 hours and subsequently in distilled

water for another 24 hours to remove excess of unbound proteins. It was then dried at 37°C after keeping a wet filter paper strip on its surface. When dried, the paper strip was removed and slides were stained with amido black for 15 minutes. Destaining was done first with decolouriser I for 20 minutes and subsequently with decolourizer II for another 20 minutes. The dried slides were made permanent by mounting with DPX mountant.

Counter immuno electrophoresis (CIE)Agarose gel

Agarose - 0.8 g

Tris borate buffer - 100 ml

Method

CIE was done by the procedure described by Talwar, (1983).

Two rows of wells were made on the slides containing agarose gel. The distance between rows was 6 mm. The wells near the anode were charged with antiserum and those near the cathode were charged with antigen of different isolates. A drop of bromophenol blue was placed in the antigen well.

Electrophoresis was done with tris borate buffer at 12 mA/slide. Electrophoresis was stopped when the dye reached the antiserum well. After 10 minutes the slides were examined for any precipitation line between the antigen and antiserum well and then stained as in AGDT.

Immunoelectrophoresis

Agarose was prepared as described for CIE and poured on to 3" x 3" glass plates. Five wells and three troughs were cut in gel as described by Talwar (1983). The wells were filled with bursal extracts from chicks infected with the four field isolates and the vaccine strain along with a drop of bromophenol blue as indicator. The slide was then placed in the electrophoresis chamber containing Tris bonate buffer. Electrophoresis was done from cathode to anode using 3 mA/slide till the dye reached the other end of the slide. At the end,the electrophoresis was stopped, the slide was removed from the trough and charged with antiserum. The anitserum was allowed to diffuse for 24 hours at room temperature in the electrophoresis chamber itself. The gel was then washed and stained as for AGDT.

Results

RESULTS

Details of IBD outbreak were obtained from various government and private poultry farms in Kerala. Four isolates of IBDV obtained from these farms were subjected to detailed study.

Unvaccinated flock

An outbreak of IBD in an unvaccinated flock in a private farm in Palghat district was reported with high morbidity (95%) and mortality (71%). This outbreak was reported 2 days after Newcastle disease vaccination.

The affected birds were lethargic not taking feed, diarrhoeic with soiled vent and were very reluctant to move and died in 2-3 days time (Fig.1).

Post-mortem examination of the dead birds revealed dehydration, haemorrhages on the thigh and breast muscle (Fig.2) and at the junction of the gizzard and proventriculus (Fig.3). The bursa was enlarged and odematous (Fig.4) or haemorrhagic (Fig.5) and when opened some showed clotted blood in the lumen (Fig.6) or haemorrhagic streaks (Fig.7). The kidneys in some of the birds were highly prominent with severe congestion (Fig.8). The spleen was enlarged but the liver was apparently normal.

Fig.1 IBD affected flock

Fig.2 Haemorrhage on the leg muscles of IBD affected chick

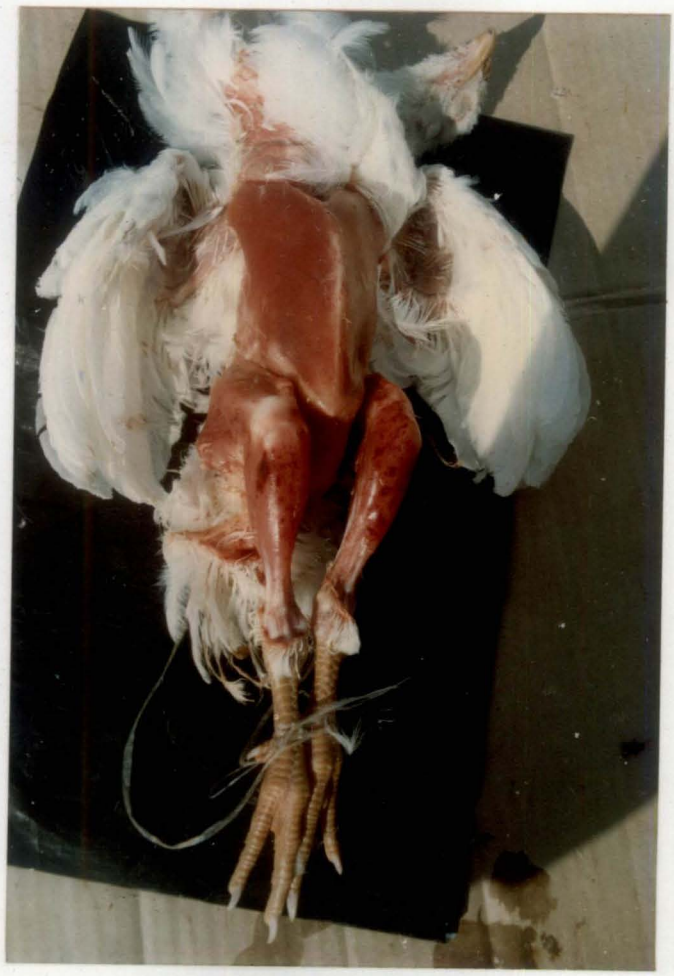
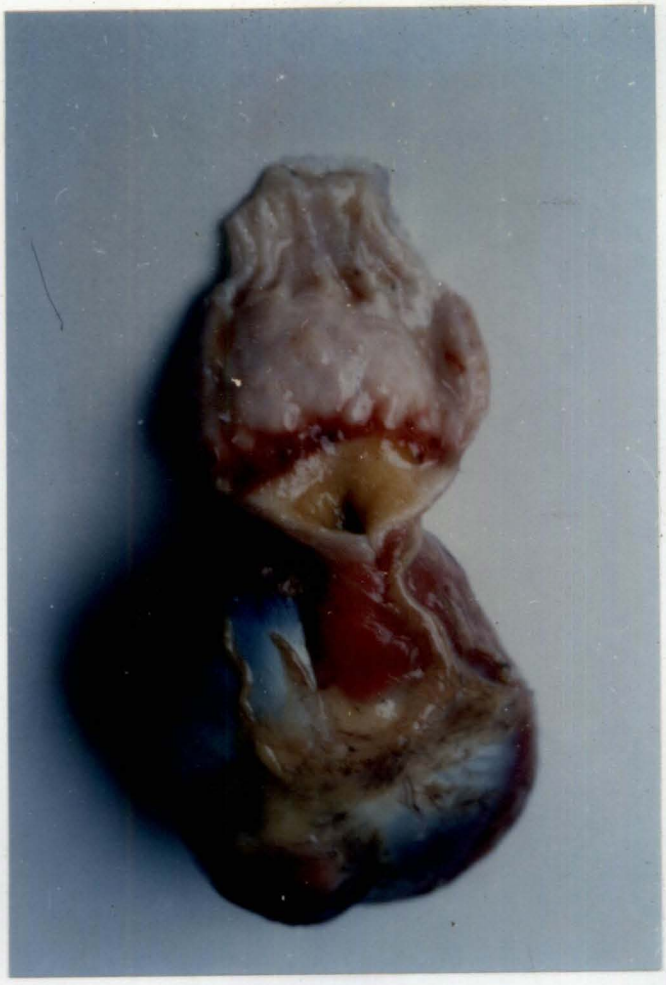


Fig.3 Haemorrhages in the mucosa of proventriculo-gizzard juncture in IBD affected chick

Fig.4 Swollen and enlarged BF in IBD affected chick



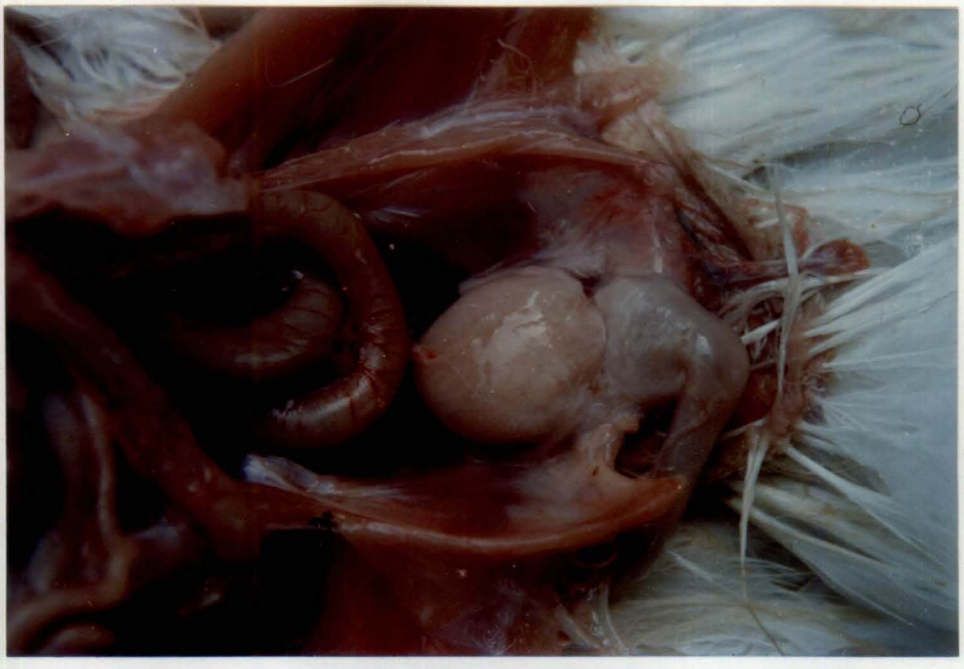
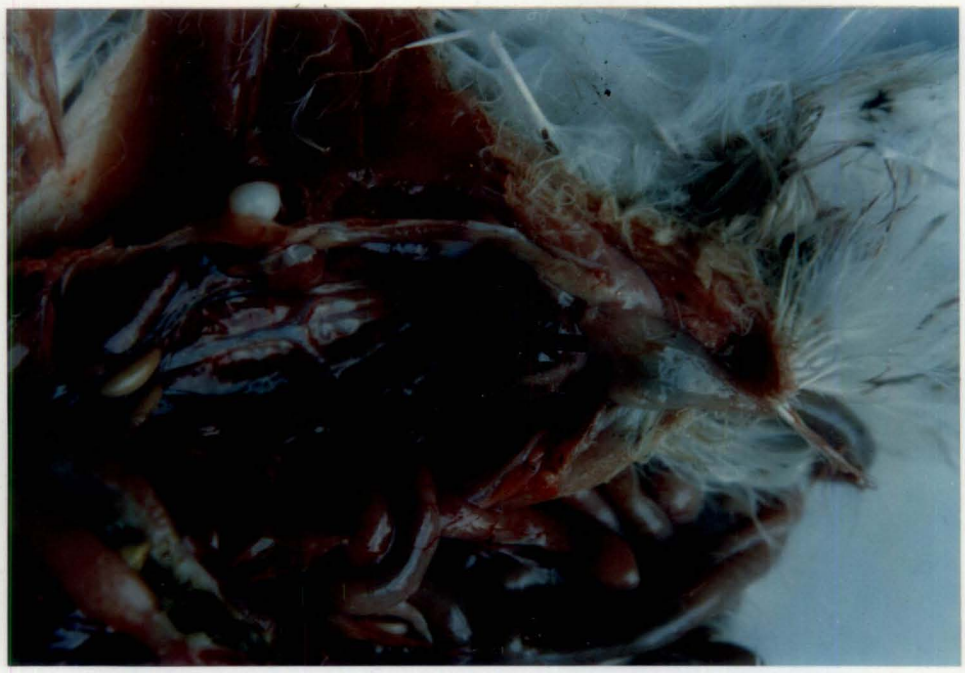


Fig.5 Haemorrhage in the BF of IBD affected chick

Fig.6. Clotted blood in the lumen of BF



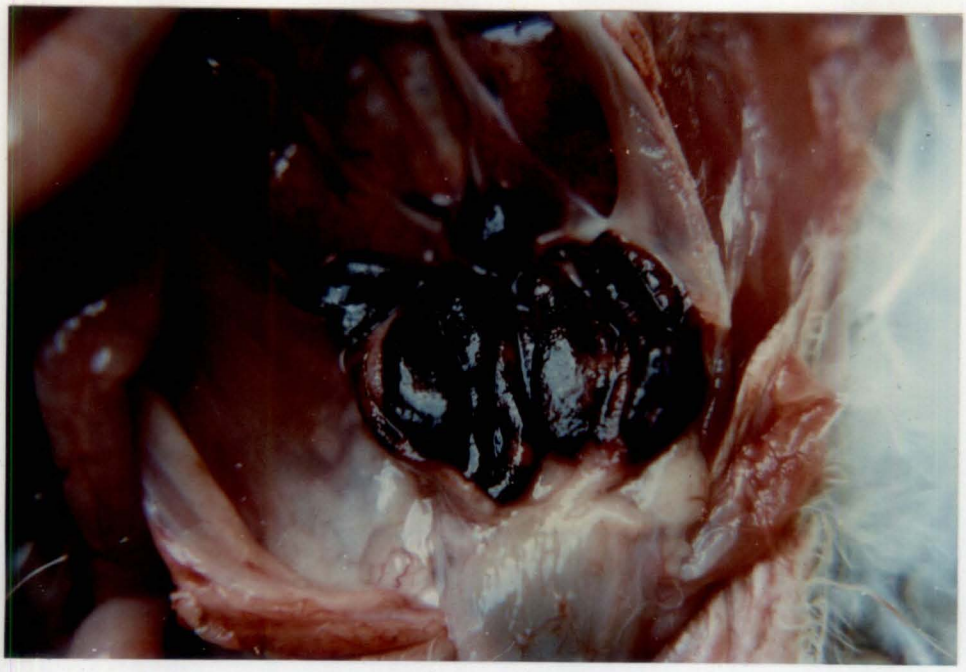
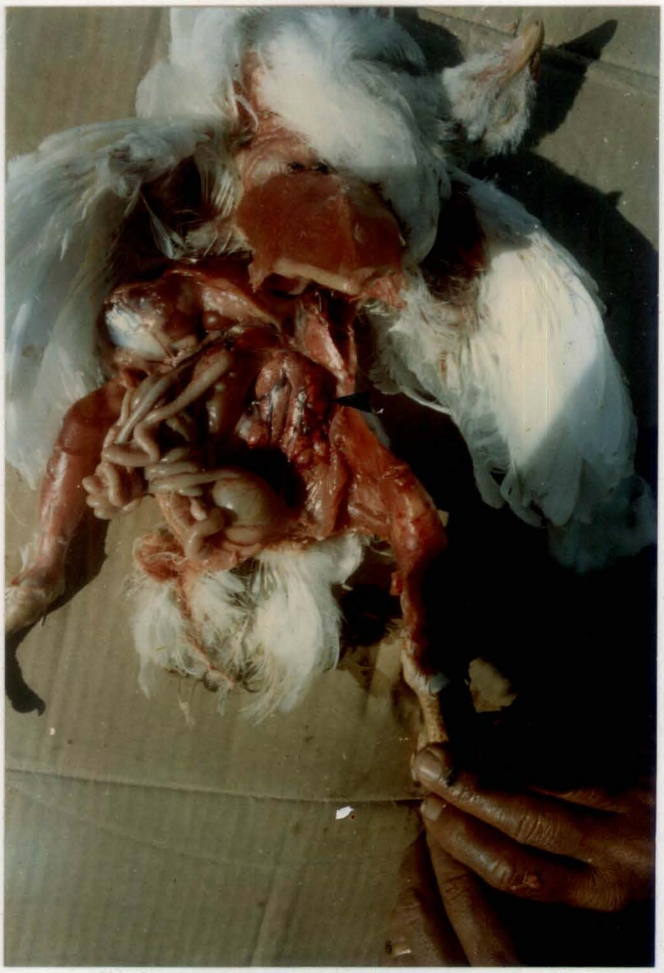


Fig.7 Streaks of haemorrhages in the lumen of BF

Fig.8 Congestion of the kidney in IBD affected chick



A second unvaccinated flock subjected to detailed investigation was from a private farm in Ernakulam. Though the birds were unvaccinated, the morbidity and mortality rates were low, the clinical symptoms and lesions were also mild compared to the outbreak described above.

Vaccinated flock

An outbreak of IBD was reported in the All India Coordinated Research Project (AICRP) for poultry, Mannuthy, during the early half of 1994. Chicks aged 7-9 weeks were mainly affected. The morbidity and mortality rates were 100 per cent and 60 per cent respectively. These chicks were from vaccinated hens and were vaccinated at the age of 21 days. The post-mortem lesions noticed were severe haemorrhages in the leg and breast muscles and annular haemorrhage at the junction in the proventriculus and gizzard. Splenomegaly, necrotic patches in the liver, enlarged bursa and congested kidney were the other observations made.

The second vaccinated flock subjected to detailed investigation from the government poultry farm, Mundayad. Here also the chicks were vaccinated by the 21st day with a live vaccine. The clinical symptoms appeared when the birds were 6-9 weeks of age. The morbidity and mortality rate were lower 60 per cent and 30 per cent respectively, compared to the outbreak in AICRP, Mannuth However, the clinical

manifestation of the affected chicks and the lesions were comparable to the birds from AICRP, Mannuthy.

Viral antigen

Bursa collected from affected chicks in the organized farms as described above and from individual cases brought to the department of Pathology for post-mortem examination were processed and tested against known positive sera. Sixty eight per cent of the bursal tissue from affected chicks were positive for IBD viral antigen by AGDT (Fig.9). Serum samples collected from birds in the convalescent state had antibodies to IBDV.

Chick embryo inoculation

Specimen collected from 4 different farms (mentioned earlier) were used for isolation of virus by the chorio allantoic membrane inoculation of 11 day old embryonated eggs. Mortality of the inoculated eggs were not noticed, especially during early passage. Mortality started by day three of the 4th passage and the rate increased in proportion to the number of passages (table 2). The predominant changes noticed were cutaneous haemorrhages all over the body (Fig.10). Congestion, oedema and thickening of the CAM were also evident (Fig.11). The bursa was enlarged and the liver

Fig.9 Screening of field cases for IBDV antigen

Diagramatic representation

O - Antiserum

I - Negative control

II - Positive Control

III-VI - Field samples

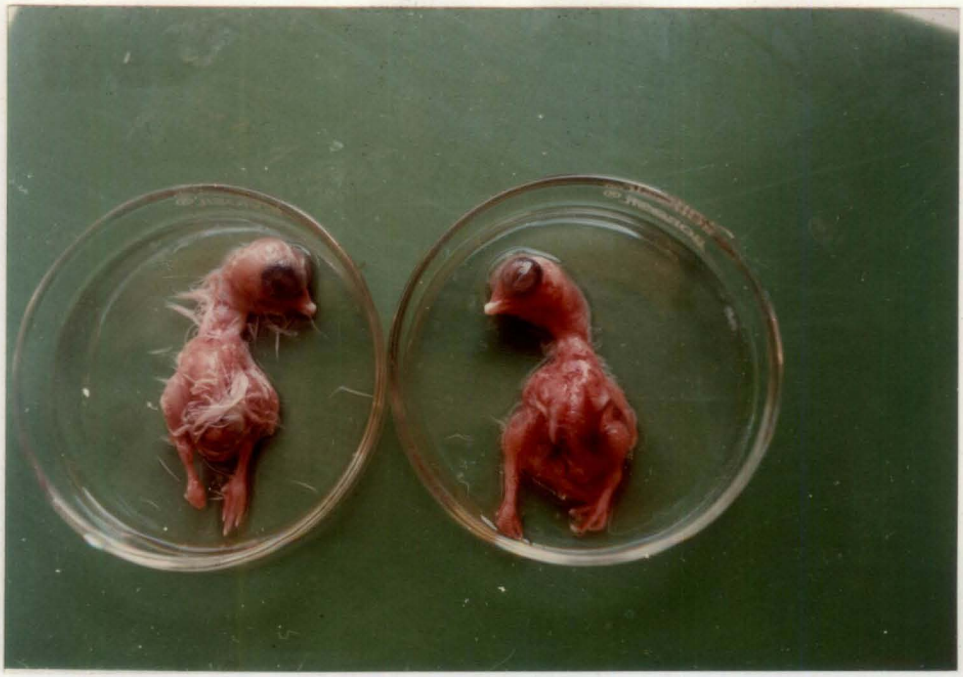
I
VI
II
O
V
III
IV

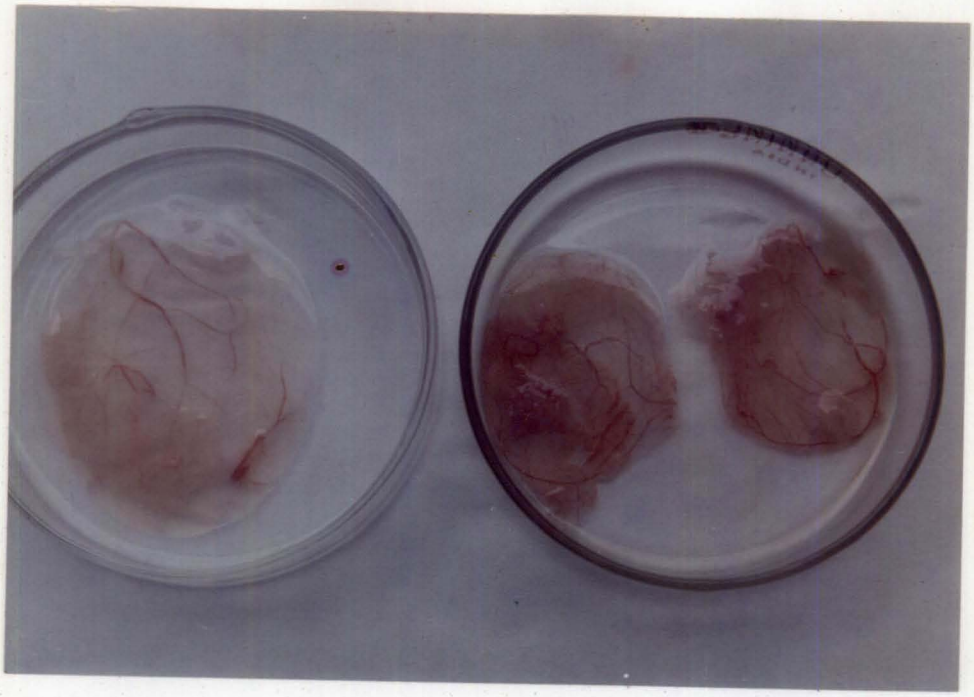
Fig.10 Cutaneous haemorrhages in the IBDV infected chick embryo

A. Normal B-infected

Fig.11 Congestion and thickening of CAM at the site of inoculation of IBDV

A. Normal B-infected





had typical yellowish green discolouration with brown patches all over.

Table 2. Embryo mortality caused by different isolates

Isolates	No. infected	Mortality					Total mortality	Mortality percentage
		Day 1	Day 2	Day 3	Day 4	Day 5		
PKD	4	-	-	1	2	-	3	75
EKM	4	-	-	-	2	-	2	50
THR	4	-	-	-	1	1	2	50
KAN	4	-	-	-	1	2	3	75
VAC	4	-	-	2	1	1	4	100
Control	4	-	-	-	-	-	Nil	Nil

The mortality rate of the infected embryos inoculated with different specimens ranged between 50-100 per cent (table 1).

Cell culture

A satisfactory monolayer of chicken embryo fibroblast was formed in 24 hrs following seeding. The morphology of the cells were of fibroblastic type. They were spindle shaped and in areas of high density, the cells were arranged with their long axis parallel to one another.

Cytopathic changes were not evident during the first and second passages. But all the isolates produced changes

in the monolayer from the third passage onwards. Initially the changes were rounding of cells in the periphery of the monolayer. This was evident from 48 hrs onwards which later on spread to the whole of the monolayer (Fig.12). Complete detachment of the cells from the substrate was evident by the sixth day. None of the cell culture passaged samples showed positive AGDT reaction.

Experimental chicks

Experimental birds that received different isolates of IBDV such as PKD, EKM, THR and KAN showed only mild clinical symptoms on the third day. The symptoms noticed were lethargy ruffled feather, depression and non inclination to move. A total of five chicks from the first four groups died on the 3rd day but further mortality could not be assessed as these chicks were destroyed on the 4th day itself.

None of the chicks that received the Lukert strain of IBD showed any clinical symptoms, so also the control birds. Observations were not made beyond three days, as they were sacrificed on that day itself.

Birds in the first four groups, when examined in detail, showed a moderately swollen gelatinous bursa with slight haemorrhage in some of them. Haemorrhages in thigh

Fig.12 CEF showing CPE following IBDV infection

and breast muscle were also evident. No specific lesions, except moderate enlargement of the bursa, was noticed in the birds that received the vaccine strain. The internal organs of all the control groups were apparently normal.

Structural proteins

Partially purified IBD virus when subjected to protein analyses by SDS-PAGE, the various polypeptides of the virus got separated into different bands. These bands were visible when stained with coomassie blue. The electrophoretic pattern of the viral proteins of different isolates are presented in (Fig.13). The molecular weights of these proteins were calculated by comparing the distance migrated by known molecular weight proteins, chymotrypsin and Bovine serum Albumin (Fig.14 and 15).

It is evident from the picture that all the isolates PKD, EKM, KAN and THR resolved nine polypeptides. The molecular weights of these proteins for all the field isolates were the same. They were 86 KD (VP_2), 77 KD (VP_4), 73 KD (VP_5), 62 KD (VP_6), 52 KD (VP_7), 47 KD (VP_8), 39 KD (VP_{10}), 36 KD (VP_{11}) and 32 KD (VP_{12}).

Fig.13 SDS-PAGE pattern of IBDV

Diagramatic representation

I - Protein marker

II - PKD (Unvaccinated)

III - EKM (Unvaccinated)

IV - THR (Vaccinated)

V - KAN (Vaccinated)

VI - VAC (Vaccine strain)

VII - NBS (Normal bursal suspension)

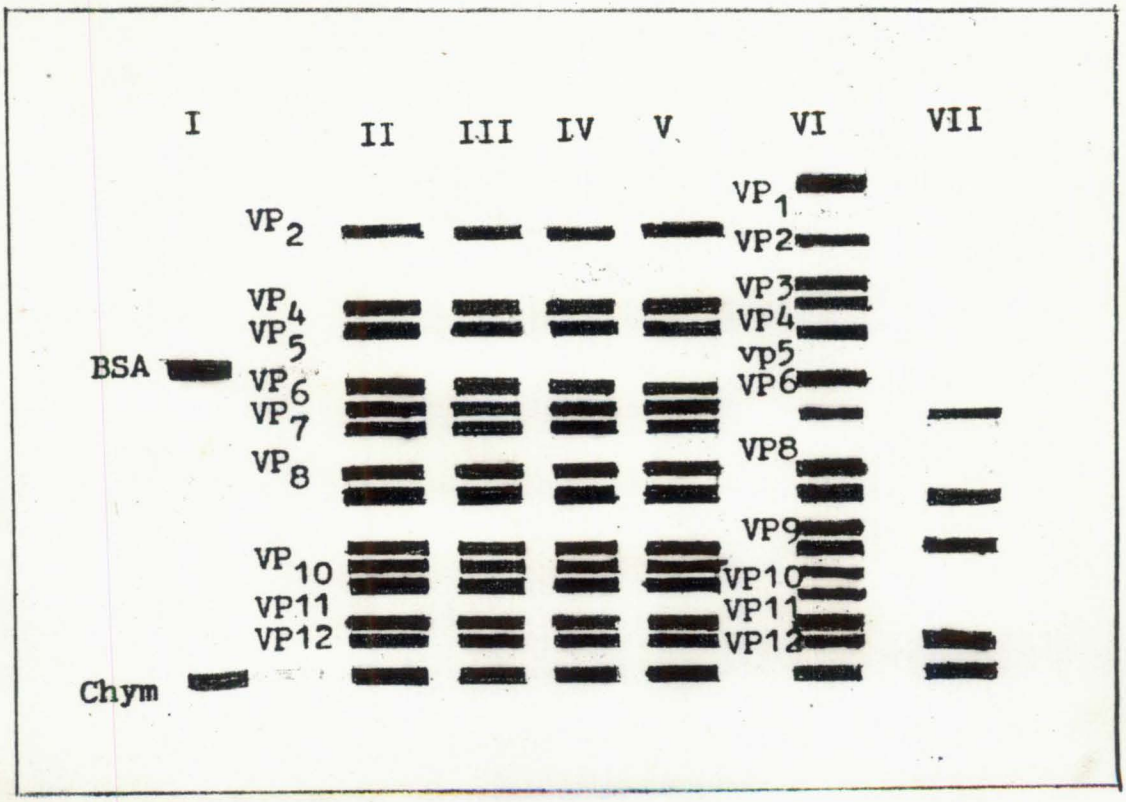

I
II
III
IV
V
VI
VII
VP2
VP4
VP5
BSA
VP6
VP7
VP8
VP10
VP11
VP12
Chym
VP1
VP2
VP3
VP4
VP5
VP6
VP8
VP9
VP10
VP11
VP12

FIG.14. MOLECULAR WEIGHT DETERMINATION OF IBDV PROTEINS (FIELD ISOLATES)

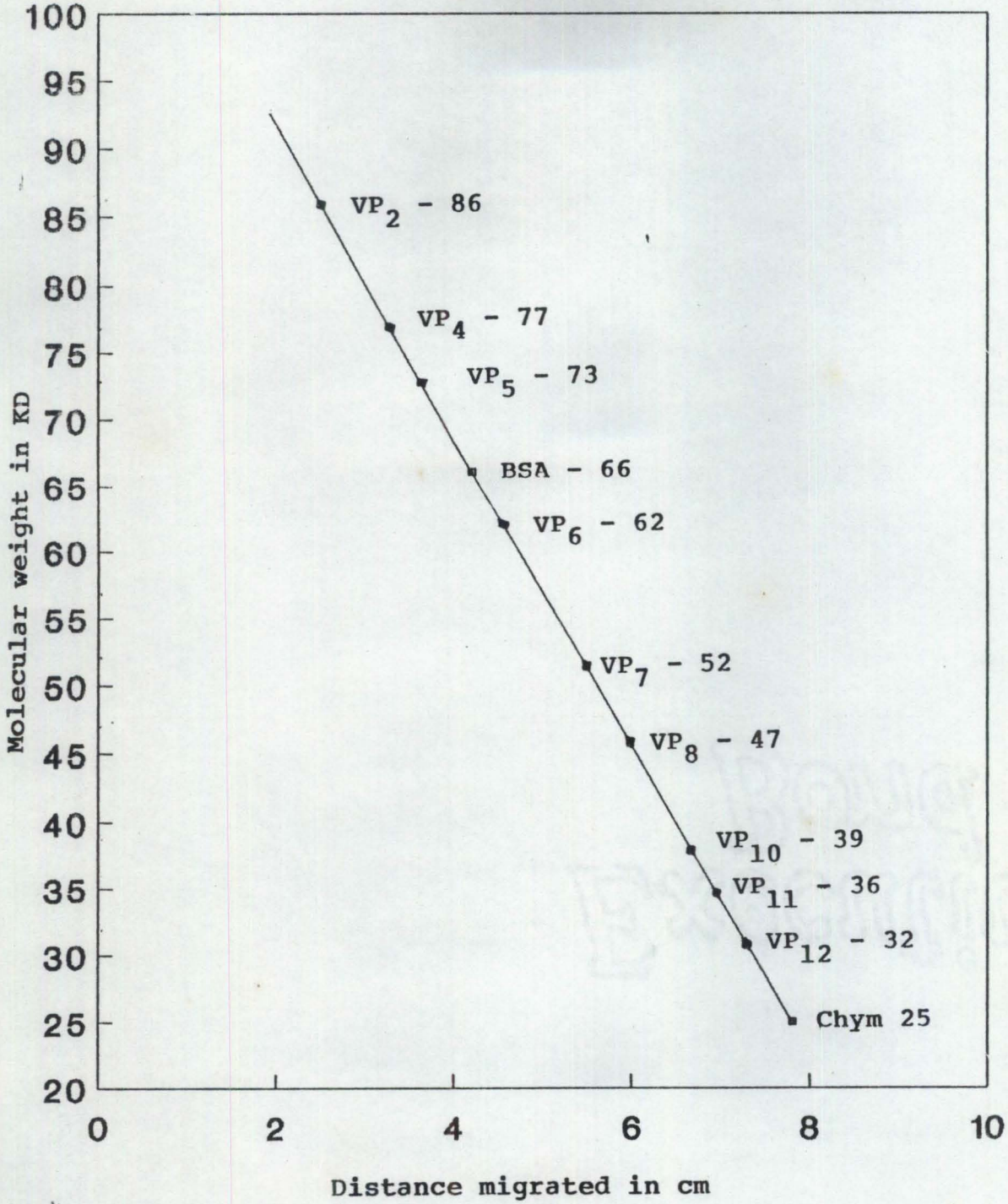


FIG.15 MOLECULAR WEIGHT DETERMINATION OF IBDV PROTEINS (VACCINE STRAINS)

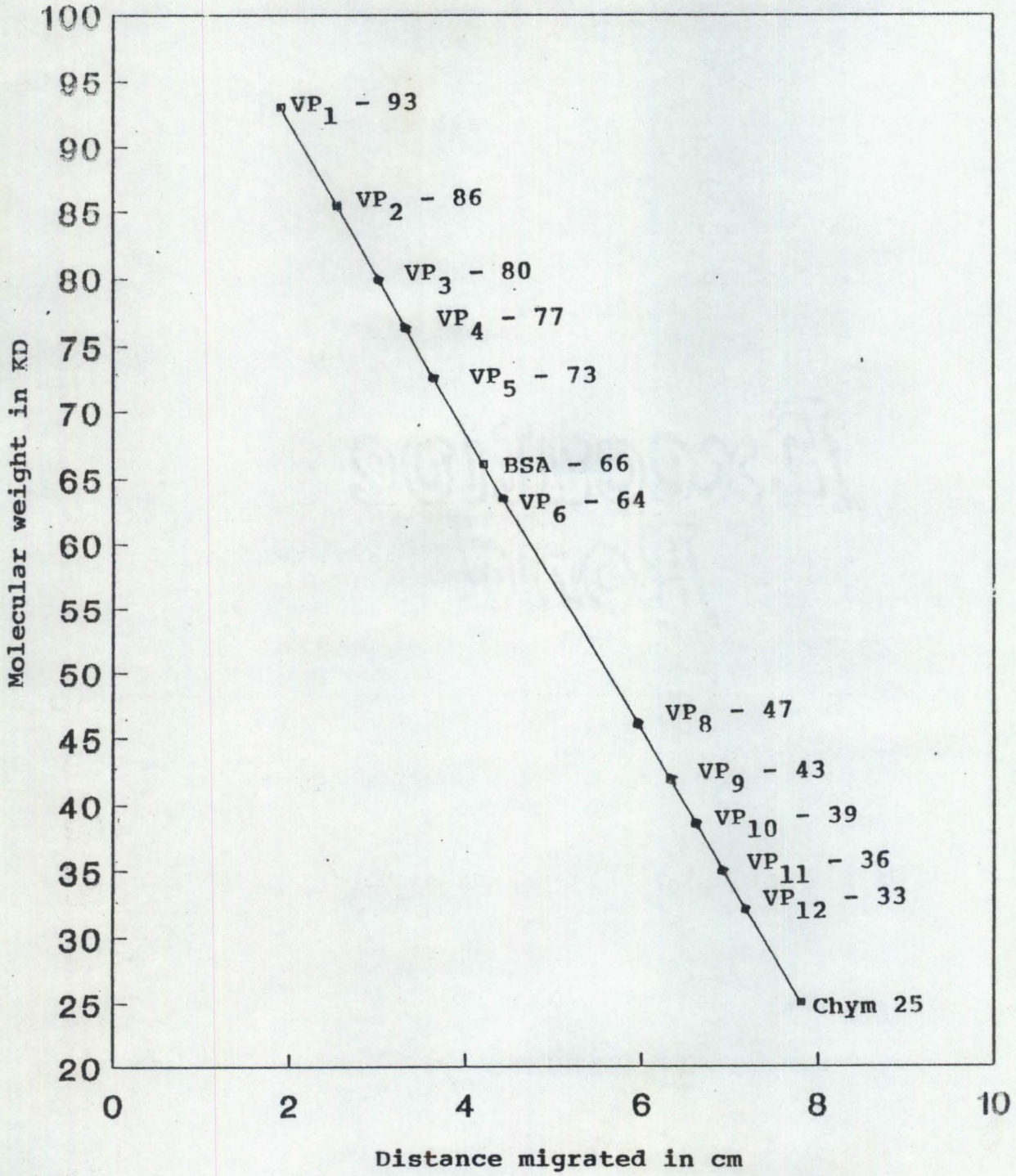


Table 3. Molecular weights of different protein fractions of infectious bursal disease virus

Protein fractions	Molecular weight in KD	
	Field isolates	Vaccine strain
VP_1	-	93
VP_2	86	86
VP_3	-	80
VP_4	77	77
VP_5	73	73
VP_6	64	62
VP_7	52	-
VP_8	47	47
VP_9	-	43
VP_{10}	39	39
VP_{11}	36	36
VP_{12}	32	33

The fifth strain VAC had eleven proteins having the following molecular weights 93 KD (VP_1), 86 KD (VP_2), 80 KD (VP_3), 77 KD (VP_4), 73 KD (VP_5), 64 KD (VP_6) 47 KD (VP_8), 43 KD (VP_9), 39 KD (VP_{10}), 36 KD (VP_{11}) and 33 KD (VP_{12}).

The molecular weights of different proteins of the IBDV studied are presented in Table 3.

Nucleic acid

Nucleic acid precipitated from partially purified IBD viral isolates from different sources and from the known strain were analysed by electrophoresis on 0.8 per cent agarose. The RNA from each isolate was separated into two distinct bands, indicating the bisegmentation of the viral RNA (Fig.16). Bursal tissue processed in the same way as infected bursae and subjected to electrophoresis did not show any line as in the case of infected bursae.

Antigenic relationship between isolates

All the five isolates were compared by AGDT using antiserum raised against vaccine strain. All the five isolates showed a sharp precipitation line with known antiserum and all showed lines of identity (Fig.17).

Counter immunoelectrophoresis

Counter immunoelectrophoresis also showed only one line of precipitation in all the five strains studied when tested against antiserum raised against vaccine virus (Fig.18).

Fig.16 Agarose gel electrophoresis pattern of IBDV RNAs

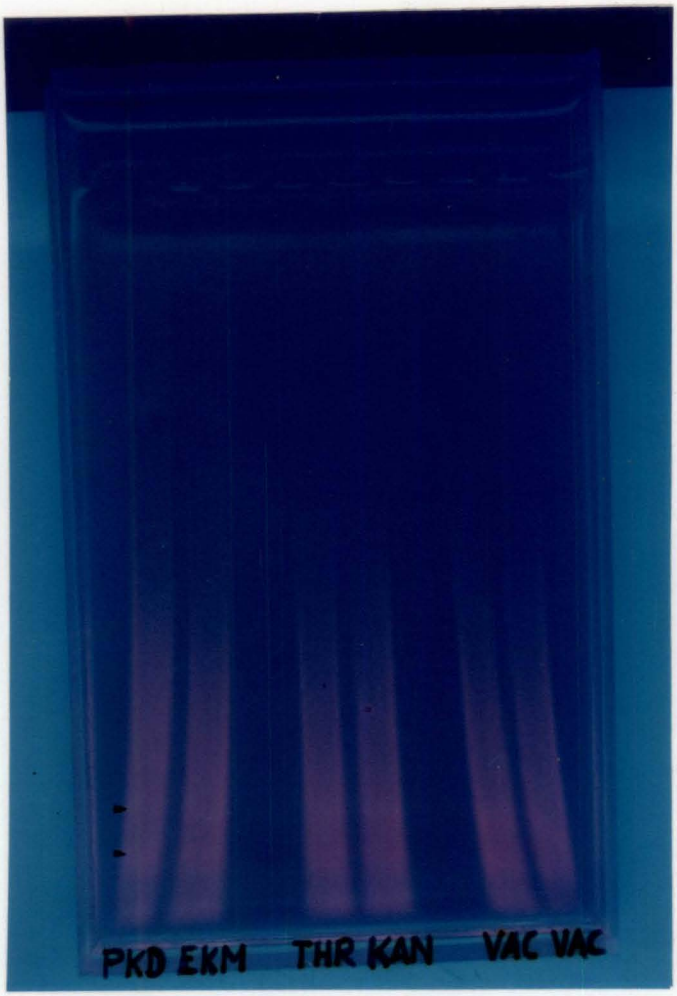

PKD EKM
THR KAN
VAC VAC

Fig.17 Antigenic relationship in AGDT

Diagramatic representation

Antigen

I - PKD
II - EKM
III - THR
IV - KAN
V - VAC
O - Antiserum against field isolate

I
II
III
IV
V
VI

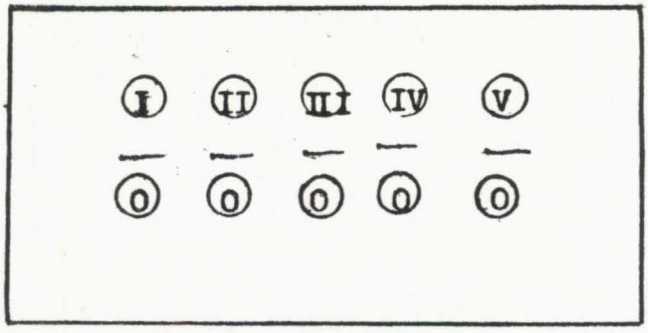
I
II
III
IV
V
O

Fig.18 Counter immuno electrophoresis showing relatedness of different isolates of IBDV

Antigen

I - PKD
II - EKM
III - THR
IV - KAN
V - VAC
O - Antiserum against field isolate


I
II
III
IV
V
O
O
O
O
O


I
II
III
IV
V
O
O
O
O
O

Immunoelectrophoresis

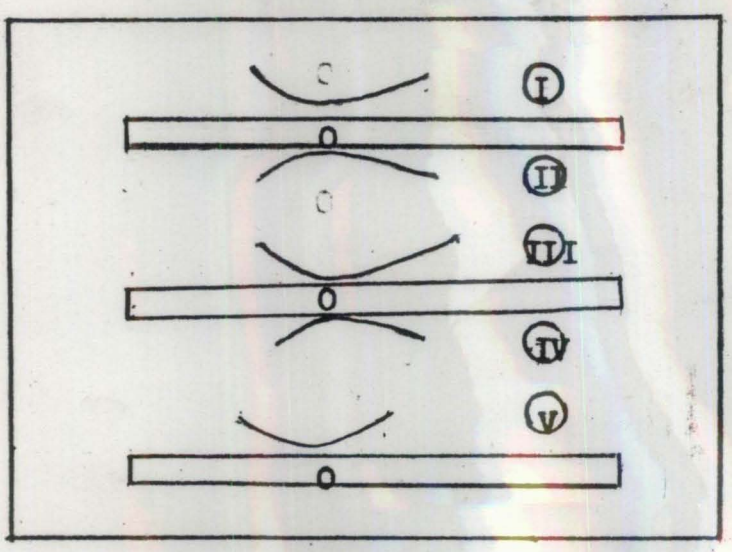
The antigenic fractions of different isolates of IBDV were studied by immunoelectrophoresis. All the five isolates showed only single arc of precipitation at the same position, towards the anode (Fig.19).

Fig.19 Immuno electrophoresis of different isolates of IBDV

Antigen

I - PKD
II - EKM
III - THR
IV - KAN
V - VAC
O - Antiserum against field isolate


I
II
III
IV
V


I
II
III
IV
V

Discussion

DISCUSSION

Infectious bursal disease is a wide spread contagious, acute or subclinical disease of young chicks caused by a birnavirus. This virus destroys the lymphoid organs, particularly the BF, resulting in reduced response to vaccines and increased susceptibility to clinical infections.

The disease is said to cause economic losses either directly or indirectly. The direct losses are on account of increased morbidity, reduced growth rate and increased rate of carcass condemnation due to marked haemorrhages in muscles (Verma et al., 1990). The indirect losses occur by way of vaccinal failures (Ajinkaya, 1980; Verma et al., 1981; Panisup et al., 1983); increased susceptibility of the chicks to environmental pathogens and persistence of the virus in an affected area due to its high resistance to routine disinfection practices (Verma et al., 1990). High mortality rate and vaccination break downs are also attributed to emergence of new strain (Jackwood and Saif, 1987).

During the present study, a detailed investigation was carried out initially to assess the gravity of this disease in Kerala. The investigation was extended to both unvaccinated and vaccinated flocks.

The clinical symptoms shown by birds in either group were lethargy, anorexia, diarrhoea resulting in soiled vent

and dehydration and sudden death in 2-3 days time. The symptoms of IBD described by other workers (Cosgrove, 1962; Cho and Edgar, 1972) were also the same as the observations made in this study. Though the clinical symptoms were the same in all the four flocks subjected to detailed study, a difference in the severity of the outbreak was evident between flocks. Severity of a disease, particularly IBD, depends on various factors such as immune status, age of the birds affected, pathotype or serotype of the virus, type of vaccines used and whether the disease is endemic or not. Of the two unvaccinated flocks - PKD and EKM - the percentage of morbidity and mortality were 90 and 70 respectively for PKD and 50 and 30 for EKM. Flock EKM had earlier outbreaks of this disease while in PKD this was the first incidence. Thus in the former case the persistence of the virus in the same area might have caused a reduction in the virulence of the virus. It is also possible that in PKD the birds might have been exposed to more of secondary infections.

A difference in the morbidity and mortality rates were also noticed between the two vaccinated flocks - THR and KAN. In the former the morbidity and mortality rates were 100 per cent and 60 per cent respectively, while in later this was only 60 and 30. In both the cases, a live virus vaccine was used for vaccination when they were at 21 days of age. In both cases chicks of 6-9 weeks age were affected.

The possible reasons for the severity of the outbreak in THR could be the high susceptibility of the birds as they are the high producing White Leghorn line (ILM 90). In this farm the last outbreak occurred in 1990 and since then the disease was being brought under control by regular vaccination. Hence the recent outbreak could be due to introduction of a virulent strain or emergence of a different pathotype strain from the persisting virus which is maintained in the farm by a low level of subclinical infection of immune birds.

Though the morbidity and mortality due to IBD was described to be very low (Cosgrov, 1962), Landgraf et al. (1967) and Jayaramiah and Mallick (1975) have reported a high morbidity rate. Mohanty et al. (1971) and Verma et al. (1990) observed a higher percentage of morbidity, upto 80 per cent. They attributed this high morbidity rate to concurrent infection with other viral and bacterial infections. High morbidity and mortality rates in IBD have also been associated with a highly virulent strain of the virus (Chettle et al., 1989; Stuart, 1984; Nunoya et al., 1992).

Macroscopic lesions of dehydration, haemorrhage in the thigh and breast muscles and at the junction of the gizzard and proventriculus, enlarged and oedematous bursa with or without haemorrhages were evident in all the four outbreaks. Congestion of the kidney and/or spleenomegaly were evident only in some birds. Similar observations were also

recorded by other workers (Cosgrove, 1962; Cho and Edgar, 1972; Verma et al., 1981 and Lukert and Hitchner, 1984).

The bursal homogenates from the four different sources were tested against known IBD antiserum for specific antigen by AGDT. Only one precipitation line was formed with all the four isolates and with the reference IBD antigen. The precipitation lines formed against all the five antigens were identical, indicating the identity of the field isolates to the reference IBD antigen. This correlates well with the observations of Ide and Stevenson (1973), Wyett and Cullen (1976) and Chauhan et al. (1980), thus confirming that the outbreaks in the above four flocks were due to IBDV.

Though various serological tests such as SNT, ELISA, IF and FA have been described for specific identification of this virus, AGDT has been extensively used for this purpose because of its simplicity (Hirai et al., 1972; Weisman and Hitchner, 1978; Verma et al., 1981).

Isolation of IBD virus in chick embryo is usually done by chorioallantoic membrane route of inoculation (Hitchner, 1970; Rao et al., 1979; Amiyakumar et al., 1991) and this technique was followed in this study also. Bursal homogenates from birds showing lesions of IBD in the four different outbreaks were used for this purpose. Embryo mortality in the range of 50 to 100 per cent was noticed

between the third and fifth day of inoculation was noticed from the fourth passage onwards. This finding similates the observations made by Saiju et al. (1990). However, Mohanty et al. (1971) observed only very low mortality. Rao et al. (1978) and Vijayapraveen et al. (1995) opined that the mortality rate in infected chick embryos might increase proportional to the number of passages the virus has undergone in this system. Mekkes and deWit (1994) opined that though variant strains of IBDV could be isolated in chick embryos, it might not kill the embryos all the time.

Hitchner (1970) and Rao et al. (1978) described the lesions of IBD virus infected embryos as congestion, oedema, thickening of the CAM and yellowish green discolouration of liver with necrotic patches. Similar observations were made in this study also.

In chicken embryo fibroblast cultures the IBD viral isolates produced cytopathic changes only from the third passage onwards. The cytopathic changes were initial rounding of cells with subsequent detachment from the substrate. Singh and Dhawedkar (1992) also described similar changes in chicken embryo fibroblasts infected with IBD virus.

Though various cell culture systems such as chicken kidney cells (Landgraf et al., 1967; Koster and Paulson, 1971; Mandelli et al., 1972; Lukert and Davis, 1974), chicken

embryo bursal cells (Lukert and Davis, 1979), Japanese quail cell line (Dobos, 1979) lymphoblastoid cell lines LSCC-BKS and LSCC-CUTO and certain mammalian cell systems (Jackwood and Saif, 1987) were used for propagation of IBD virus, chicken embryo fibroblast is considered as the cell culture system of choice for IBD virus propagation (Petek et al., 1973; Nick et al., 1976) and used by various workers (Muller et al., 1979; Skeels and Lukert, 1980; Alamsyah et al., 1992).

McFerran et al. (1980) expressed the difficulty in using cell cultures for the isolation IBDV from field cases. Similarly Mekkes and deWit (1994) also opined that though cell cultures could be used for isolation of less pathogenic strains, virulent strains were difficult to get adapted in this system.

Four week old chicks that received the field isolates had mild clinical symptoms, which were absent in the age matched chicks that were infected with the vaccine strain. Postmortem examination of these birds on the third day of infection revealed macroscopic lesions in chicks infected with the field isolates. The lesions were moderate swelling of the bursa and haemorrhages in the thigh and breast muscles in few cases. In chicks that received the vaccine virus, the lesions were very mild and confined only to the bursa. Appearance of clinical symptoms in 2-4 days time following

different routes of infection of experimental chicks have been reported by Helmbolt and Garner (1964); Landgraf et al. (1967), Wood et al. (1981); Schneider and Hass (1967), Lange et al. (1981) and Singh and Dhawadhar (1994).

Though the field isolates were from outbreaks showing morbidity and mortality ranging from 50-100 per cent (PKD and EKM) and 30-80 per cent (THR and KAN) respectively, such a severity was not noticed when these isolates were used for experimental infection of 4 week old chicks. This very well explains the fact that IBDV alone cannot produce high morbidity and mortality in chicken. It is the secondary infections, either bacteria or virus, that affect the already immunodeficient chicks coupled with poor management practices that lead to severe outbreak resulting in high morbidity and mortality. All these factors are well controlled in the experimental infection and chicks manifested only mild clinical symptoms and the lesions were mainly confined to the bursa only. The presence of IBD viral antigen in the bursa of the experimentally infected birds show that they had picked up infection and the virus had multiplied in this organ.

Mild symptoms and very low mortality rate as observed in this study was also reported by Mohanty et al. (1981). However high rate of mortality, upto 100 per cent, with peak virus titres in the bursa within two days after inoculation

of chicks with CU strain of IBD virus was reported by Kaufer and Weiss (1980). Mild microscopic changes such as hyperaemia of the kidney, thymus and caecal tonsils and depletion of lymphoid cells in the spleen without producing clinical signs and mortality were observed by Dongavonkau and Rao (1979), Verma et al. (1981) and Panisup et al. (1989), though Mohanty et al. (1984) reported 5-20 per cent mortality and 80 per cent morbidity with the same isolate.

Structural proteins

Four field isolates and one vaccine strain of IBD virus were concentrated and partially purified to study the protein and nucleic acid profile of this virus.

The structural proteins were analysed in SDS-PAGE using 9 per cent acrylamide.

All the field isolates and the vaccine strain were found to have nine polypeptides, with the same molecular weights namely 86 KD (VP_2); 77 KD (VP_4); 73 KD (VP_5), 62 KD (VP_6), 52 KD (VP_7), 47 KD (VP_8), 39 KD (VP_{10}), 36 KD (VP_{11} AND 32 KD (VP_{12}). Unlike the observations made in this study, Nick et al. (1976), Kibenge et al. (1988) and Muller et al. (1985) demonstrated four polypeptides using the same system for protein analyses. The molecular weights of these fractions were 110 KD: 50 KD, 35 KD and 25 KD respectively for VP_1, VP_2,

VP_3 and VP_4. Dobos (1979) also resolved four polypeptides but the molecular weighTs were 90 KD, 41 KD, 35 KD and 28 KD, with an additional protein of molecular weight of 47 KD. Variations in the number and molecular weights of protein fractions of IBDV have also been reported by Hirai et al. (1979). The molecular weight of the IBD viral protein resolved by Hirai (1979) ranged between 133 KD and 23 KD. A total of nine proteins had also been reported (Muller and Becht, 1982). Jackwood et al. (1984) opined that the two serotypes can be distinguished by their structural proteins when they were grown in CEF cultures. They observed that serotype II lacked VP_2 (42 KD) and a difference in the molecular weight of VP_3 and VP_4. However no mention about the differentiation of subtypes of serotype I is seen in any of the published reports.

Seven polypeptides resolved by Kher (1988) had molecular weight of 82 KD, 74 KD, 44 KD, 37 KD, 30 KD, 28 KD and 26 KD. The seven polypeptides reported by Vijayapraveen et al. (1995) were of 97, 56, 53, 50, 45, 29 and 25 kilodaltons. Variation in the number and molecular weight of polypeptides of IBDV resolved by various workers can be attributed to the change in the methodology adopted, source of the sample and the degree of purification attained. It is worth mentioning here that all the field isolates had the same pattern (number and molecular weight) of structural

protein, which was absent from the uninfected sample processed in the same way as that of infected sample. Hence all the nine bands can be considered as specific for IBDV and not due to contamination.

The vaccine strain resolved 11 peptides of 93 KD (VP_1), 86 KD (VP_2), 80 KD (VP_3), 77 KD (VP_4), 73 KD (VP_5), 62 KD (VP_6), 47 KD (VP_8), 43 KD (VP_9), 39 KD (VP_{10}), 36 KD (VP_{11}) and 33 KD (VP_{12}). The VP_1, VP_3 and VP_9 were absent in the field isolates whereas VP_7 was missing in the vaccine strain. A difference in molecular weights of VP_6 and VP_{12} were also detected. These differences in the structural proteins can be considered as a distinguishing feature, as the virus samples were subjected to the same method of processing and electrophoresed in the same gel. Vijayapraveen et al. (1955) also reported that the vaccine strain differed from the field strain as the former lacked VP_7 (26 KD). However no mention is seen in his paper regarding the vaccine strain used in his study.

Nucleic acid

Nucleic acids precipitated from the partially purified IBD viral isolates, from unvaccinated and vaccinated flocks and the vaccine strain, were subjected to electrophoresis in 0.8 per cent agarose gel. The RNA of all the viral strains got separated into two distinct

bands, indicating the bisegmentation of the viral RNA. This observation is similar to the findings of Muller et al. (1979); Dobos et al. (1979b), and Lange et al. (1987). Muller et al. reported that the molecular weights of the two segments of the RNA were 2.2×10^6 and 2.5×10^6. No attempt was made in the present study to calculate the molecular weight of the two segments of the RNAs of various strains. However the migration pattern of RNAs of all the field isolates and the vaccine strain were comparable and no difference was noticed. Though the migration pattern of viral RNA segments help in the differentiation of serotypes of IBDV (Jackwood et al., 1984; Becht et al., 1988), a difference in the migration pattern of RNAs within same serotype has not so far been reported, which is in agreeement with the finding of the present study.

Antigenic relationship between isolates

Serological tests such as AGDT, CIE and IE were used to identify the antigenic constituents and the relationship between the field isolates and the vaccine strain. All these test revealed only one precipitation line which was identical for all the antigens. As against the findings reported here, Verma et al. (1990) observed 1-3 precipitation lines in 18-24 hrs time by AGDT. Immunoelectrophoresis is said to be superior to other tests to study the number of antigenic

components. The formation of a single line of precipitation with all the five isolates showed that there is only one immunogenic component in all the five IBDV strains tested, confirming their antigenic identify. Becht et al. (1988), Jagadish et al. (1988) and Fashey et al. (1989) reported that though IBDV possess four or more structural proteins, only VP_2 has been identified as the major host protective antigen.

McFerran et al. (1980); Wyeth and Cullen (1988); Singh and Dhawedker (1993); used this test to detect the antigenic relationship between two serotypes of IBDV and for comparison of local isolates with standard strain for antigenic variation.

Results of analyses of proteins and nucleic acids of the isolates from unvaccinated and vaccinated flocks and the vaccine revealed similarity of these strains. The difference in the structural proteins of the field isolates from that of the vaccine strain was not evidenced in the serological tests. This may be due to the fact that the antiserum used in these tests were raised against the field isolates, while the vaccine strain used was the Lukert strain.

Following are the conclusions made during this study.

Outbreaks of IBD are often reported in flocks vaccinated either with an avirulent or intermediate strain.

Agar gel diffusion test, counter immunoelectrophoresis and immunoelectrophoresis have shown that the isolates (two each from unvaccinated and vaccinated flocks and the avirulent vaccine strain) did not have any difference in their antigenic constituents.

Both the isolates, two each from unvaccinated and vaccinated flock, did not differ in their pathogenicity, as evidenced by experimental infection studies.

All the field isolates had the same protein profile, both in the number of polypeptides and in their molecular weight.

The avirulent vaccine strain lacked one of the structural proteins of (VP_7 - 52 KD) of the field isolates but had three additional proteins (VP_1 - 93 KD, VP_3 - 80 KD, VP_9 - 43 KD).

A difference in the molecular weights two protein fractions (VP_6 64 KD and VP_{12} 33 KD) was also observed.

No difference, either in the number of segments or in their migration pattern, was observed with the viral RNAs.

From the above observations, break down of immunity in vaccinated flocks can be attributed to

1. Lack of uniformity in the level of maternal antibody
2. Defective handling and preservation of vaccines
3. Use of improper vaccine and vaccination.
4. Improper timing and schedule of vaccination.
5. Defective management practices.

Though antigenic difference was not evident between the field isolates and the avirulent vaccine strain, the vaccine strain had distinguishing characters in its protein profile. But before this variation is considered as one of the reasons for vaccination break down, further studies incorporating the intermediate vaccine strain, extensively used in Kerala, is required. However, from the observations made during this study, Kerala can be considered as a moderate to high risk area and it is better to use a highly antigenic intermediate or intermediate-plus (low attenuation) vaccine. In moderate to high risk areas where conventional and very virulent IBDV can be expected, the vaccination programme recommended by Lasher and Shane (1994) is as follows, administration of intermediate strain IBD virus vaccine at 12-14 days, followed by a second vaccination with either intermediate or intermediate-plus vaccine at 21-24 days. A third vaccination at 7 weeks is advisable to give additional protection. All these are to be administered in

drinking water. For parent flocks these should be followed by an inactivated oil emulsion vaccine at 18-19 weeks and again at 40-45 weeks of age, by the subcutaneous route. Only a single administration of an intermediate plus vaccine in drinking water at 8-14 days of age is recommended for broilers.

Summary

SUMMARY

Outbreaks of IBD in unvaccinated and vaccinated flocks are frequently reported from various parts of Kerala and other states. It has also been brought to notice that severity of the outbreaks varied between flocks. Hence it was felt worth while to take up a study on the structural analyses of the isolates from cases of IBD, in unvaccinated and vaccinated flocks and compare it with a reference vaccine strain. Antigenic relationship between these strains was also included in this study.

Four flocks, two each from unvaccinated and vaccinated,were subjected to detailed investigation and were used for virus isolation for the structural analyses and to studyantigenic relationship. An avirulent vaccine strain (Lukert strain) was used as the reference strain. The morbidity and mortality rates in unvaccinated and vaccinated flocks varied between 50-100 per cent and 30-70 per cent, respectively.

Virus isolated from the birds showing clinical symptoms and the vaccine strain were propagated in chick embryo by the CAM route. Embryo mortality ranging from

50-100 per cent was noticed only from the 4th passage onwards. The predominant changes were cutaneous haemorrhages all over the body, congestion and thickening of CAM,

enlargement of bursa and typical yellowish green discolouration with brown patches in the liver.

The embryo passaged virus was propagated in CEF. The CPE started from the third passage, showing initial rounding of cells followed by complete detachment of cells from the substrate. The CPE produced by all the isolates were the same. In four week old chicks the field isolates produced mild symptoms on the third day. The mortality rate was negligible chicks receiving field isolates revealed moderately swollen gelatinous bursa with slight haemorrhages and haemorrhages in thigh and breast muscle. No specific lesions were noticed in chicks that received vaccine strain, except a mild enlargement of bursa.

The virus was concentrated, partially purified and used for protein and nucleic acid analyses.

In protein analysis of different isolates by SDS-PAGE, all the four isolates resolved nine peptides each. The peptide pattern of all the field isolates were similar;they were 86 KD (VP_2), 77 KD (VP_4), 73 KD (VP_5), 62 KD (VP_6), 52 KD (VP_7), 47 KD (VP_8), 39 KD (VP_{10}), 36 KD (VP_{11}) and 32 KD (VP_{12}). The vaccine strain had eleven peptides with 93 KD (VP_1), 86 KD (VP_2), 80 KD (VP_3), 77 KD (VP_4), 73 KD (VP_5), 64 KD (VP_6), 47 KD (VP_8), 43 KD (VP_9), 39 KD (VP_{10}), 36 KD (VP_{11}) and 33 KD (VP_{12}).

Differences noticed in the protein profile of the strains studied were absence of VP_1 (93 KD), VP_3 80 KD and VP_9 (43 KD) in field isolates, absence of VP_7 (52 KD) in vaccine strain and differences in the molecular weights of VP_6 and VP_{12}.

The nucleic acid was studied by agarose gel electrophoresis. All the four isolates and the vaccine strain revealed two bands in the same position.

The antigenic relationship was studied by AGDT, CIE and IE. The AGDT produced single precipitation line for all the isolates with the standard antiserum and all the isolates showed line of identity with the reference antigen.

Counter immunoelectrophoresis also revealed single line of precipitation at the same position for all the isolates and the reference antigen. Immunoelectrophoresis of different isolates produced single arc towards the anode which was similar for all the isolates and the reference antigen.

References

REFERENCES

Abdu, P.A. (1986). Infectious bursal disease immunization failures in chicken in Nigeria. Trop. Anim. Hlth Prod. 18: 123-125.

Ajinkya, S.B. Survashe, B.D. and Sardeshpande, P.D. (1980). Breakdown of immunity to Ranikhet disease (Newcastle disease) with Infectious bursal disease in broiler chicks. Indian Vet. J. 57: 265-269.

Alamsyah, E., Dhillon, A.S. and Evermann, J.E. (1992). Comparative pathogenicity and serogrouping of three Washington isolates of infectious bursal disease virus. Avian Dis. 37: 655-659.

Allan, W.H., Faragher, J.T. and Cullen, G.A. (1972). Immunosuppression by the infectious bursal agent in chickens immunized against Newcastle disease. Vet. Rec. 90: 511-512.

Amiyakumar, and Rao, A.T. (1992). An indirect immunoperoxidase method of diagnosis of infectious bursal disease Indian Vet. J. 69: 176-177.

Amiyakumar, Rao, A.T. and Parhi, N.K. (1991). An indirect ELISA for detection of antibody in chicks infected with infectious bursal disease virus. Indian Vet. J. 68: 1109-1112.

Anjum, A.D. (1994). Outbreak of infectious bursal disease in vaccinated chickens due to aflatoxicosis. Indian Vet. J. 71: 322-324.

Anon. (1991-86). Annual reports of the Division of Avian Disease. Indian Veterinary Research Institute. Izatnagar, India.

August, J.T. and Eoyong, L. (1967). RNA virus, RNA polymerase: Detection, purification and properties. In. "Methods in Virology", Vol. III. Edited by Maramorosch, K. and Koprowski, H. Academic Press, New York, London, pp.105.

Ayoub, N.N.K. and Malek, G. (1976). Der Na Chweis des Erregers de Gumboro-disease in Agypten. Mh. Vel. Med. **31**: 106-108. (Cited by Okoye, 1984).

Azad, H.A., Barrett, S.M. and Fahey, K.J. (1985). The characterization and molecular cloning of the double stranded RNA genome of an Australian strain of infectious bursal disease virus. Virology **143**: 35-44.

Azad, A.A., Jagadish, M.N., Brown, M.A. and Hudson, P.J. (1987).Deletion mapping and expression in Escherichia coli of the large genomic segment of a Birnavirus. Virology **161**: 145-152.

Aziz, S.S. (1988). Studies on infectious bursal disease in relation to seroepidemiology, virus isolation and immunosuppression. Indian J. Poult. Sci. **22**: Suppl.I, 280-281.

Aziz, S.S. and Raghavan, R. (1986). Studies on infectious bursal disease. I. serological survey in boriler and layers in Bangalore. Indian J. Poult. Sci. **21**: 39-41.

Ba, O. and Chamoiseau, G. (1977). La bursite infectieuse virale ou maladie de Gumboro em mouritania. Bull off int Epizoot. **88**: 275-280. (Cited by Okoye, 1984).

Badiola, C., Oliver, F. and Parla, J. (1969). Enfermedad bunsal infecciosa (Entermedad de Gumboro). Laboratorios Renuidos madrid. (Cited by Okoye, 1984).

Balachandran, C., Muralimanohar, B., Sundararaj, A. and Thanickachalam, M. (1991). Infectious bursal diseaseand its association with gangrenous dermatitis in Tamil Nadu. Indian Vet. J. 68: 718-720.

Bayliss, C.D., Spies, V., Shaw, K., Peters, R.W., Papageorgiou, A., Muller, H. and Boursnell, M.E.G. (1990). A comparison of the sequence of segment A of four Infectious bursal disease virus strains and identification of a variable region in VP2. J. Gen. Virol. 71: 1303-1312.

Becht, H., Muller, H. and Muller, H.K. (1988). Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. J. Gen. Virol. 69: 631-640.

Benton, W.J., Cover, M.S., Rosenberger, J.K. and Lake, R.S. (1967). Physico chemical properties of infectious bursal agent. Avian Dis. 11: 438-445.

Berg, N.W. (1982). Rapid detection of infectious bursal disease antibodies by counter immunoelectrophoresis. Avian Pathol. 11: 611-614.

Bhattacharya, H.M., Chakravarthy, G.G. and Biswas. (1983). Gumboro disease in broiler chicks in West Bengal. Poult. Guide. 21: 61-64.

Briggs, D.J., Whilfill, C.E., Skeeles, J.D. and Reed, K.D. (1986). Application of the positive/negative ratio method of analysis to quantitate antibody response to infectious bursal disease virus using a commercially available ELISA. Avian. Dis. 30: 216-218.

Chang, C. and Hamilton, P.B. (1982). Increased severity and new symptoms of infectious bursal disease during aflatoxicosis in broiler chickens. Poult. SCi. **61**: 1061-1068.

Chauhan, H.V.S., Jha, G.J. Singh, M.P. and Thakur. (1980). Outbreaks of infectious bursal disease in poultry. Ind. J. Poult. Sci. **15**: 253-258.

Chettle, N.J. and Wyeth, P.J. (1989). Failure of maternally derived infectious bursal disease antibodies to serotype 1 and 2 to protect against heterologous virus. Br. Vet. J. **145**: 165-169.

Cheville, N.F. (1967). Studies on the pathogenesis of Gumboro disease in the Bursa of Fabricius, Spleen and Thymus of Chicken. Am. J. Pathol. **51**: 527-551.

Cho, Y. and Edgar, S.A. (1969). Characterization of the infectious bursal agent. Poult. Sci. **48**: 2102-2107.

Cho, Y. and Edgar, S.A. (1972). Characterization of infectious bursal disease. Poult. Sci. **51**: 60-69.

Cho, B.R. and McDonald. (1980). Infectious bursal disease vinus: Further characterization with evidence for a single stranded RNA virus. Avian Dis. **24**: 423-434.

Cosgrove, A.S. (1962). An apparently new disease of chicken avian nephrosis. Avian. Dis. **6**: 385-389.

Cruz-Coy, J.S., Giambrone, J.J. and Panangala, V.S. (1993). Production and characterization of monoclonal antibodies against variant A infectious bursal disease virus. Avian Dis. **37**: 406-411.

Cullen, G.A. and Wyeth, P.J. (1976). Response of growing chickens to an inactivated infectious bursal disease antigen in oil emulsion. Vet. Rec. 99: 418.

Cummings, T.S., Broussand, C.T., Page, R.K., Thayer, S.G. and Lukert , P.D. (1986). Infectious bursal disease in turkeys. Vet. Bull. 56: 757-765.

Dash, B.B., Verma, K.C. and Kataria, J.M. (1991a). Susceptibility of embryos, cell cultures and cell lines to a local isolate of infectious bursal disease virus. Indian J. Poult. Sci. 26: 89-94.

Dash, B.B., Verma, K.C. and Kataria, J.M. (1991b). Comparison of some serological tests for detection of infectious bursal disease virus infection in chicken. Indian J. Poult. Sci. 26: 160-165.

Dennett, D.P. and Bagust, T.J. (1980). The natural prevalence of antibodies to infectious bursal disease in Australian poultry flocks. Aust. Vet. J. 56: 94-95.

Devos, A., Viaene, N., Spanoghe, L. and Vanimpe, J. (1966). Comparative study of postmortem findings at Gent Poultry Clinic during 1966. Vlaams diergeresk. Tijdschr. 35: 176-184. (Cited by Okoye, 1984).

Dobos, P. (1979). Peptide map comparison of the proteins of infectious bursal disease virus. J. Virol. 32: 1046-1050.

Dobos, P., Hill, B.J., Hallett, R., Kells, D.T.C., Becht, H. and Teninges, D. (1979). Biophysical and biochemical characterization of five animal viruses with bisegmented double stranded RNA genomes. J. Virol. 32: 593-605.

Dongaonkar, V.D., Kolte, G.N. and Rao, K.N.P. (1979). Some observations on the histopathology of experimentally infected chickens with infectious bursal disease virus. Indian Vet. J. **56**: 541-545.

Dongaonkar, V.D. and Rao, K.N.P. (1979). Serological prevalence of infectious bursal disease in Madhya Pradesh and Maharashtra. Indian Vet. J. **56**: 811-815.

Dorn, P., Krorthaler, O. and Schindler, P. (1968). The course and control of infectious bursal disease. Berl. Much. Tieraztls Wschs **81**: 272-175. (Vet. Bull. **29**: 1300).

du Preez, .J.H. (1994). Control of very virulent IBD in South Africa. World Poult. December 17-18.

Durojaiye, O.Q., Adene, D.F. and Owoade, A.A. (1985). Counter immunoelectrophoresis in the diagnosis of infectious bursal disease of poultry. Trop. Anim. Hlth. Prod. **17**: 225-229.

Edgar, S.A. and Cho, Y. (1965). Avian nephrosis and its control by immunization. Poult. Sci. **44**: 1368.

Edgar, S.A. and Cho, Y. (1973). Immunization of chickens for control of infectious bursal disease. Poult. Sci. **52**: 492-497.

Fahey, K.J., O'Donnel, I.J. and Bagust. (1985). Antibody response to the 32 k structural proteins of infectious bursal disease virus neutralizes viral infectivity in vitro and confer protection on young chickens. J. Gen. Virol. **66**: 2693-2702.

Faragher, J.T. (1972). Infectious bursal disease of chickens. Vet. Bull. **42**: 361-369.

Faragher, J.T., Allen, W.W. and Wyeth, P.J. (1974). Immuno-suppressive effect of infectious bursal agent on vaccination against Newcastle disease. Vet. Rec. 95: 385-388.

Ganesan, P.I., Appaji Rao, V.N., Karunakaran, K., Elankumaran, S., Ramakrishna, J., Gunaseelan, and Raghavan, N. (1990). Diagnosis of infectious bursal disease by detection of viral antigen. Cheiron 19: 205-208.

Giambrone, J.J. and Eckman, M.K. (1980). Infectious bursal disease immunization study field trial with a commercially prepared attenuated vaccine. J. Am. Vet. Med. Assoc. 119: 258.

Giambrone, J.J., Edison, C.S. and Kleven, S.H. (1977). Effect of infectious bursal disease on the response of chickens to Mycoplasma synoviae, Newcastle disease virus and infectious bronchitis virus. Am. J. Vet. Res. 38: 251-253.

Giambrone, J.J., Yu, M. and Eckman, M.K. (1982). Field trials with an oil emulsion infectious bursal disease vaccine in broiler pullets. Poult. Sci. 61: 1823-1827.

Giron, P.C. (1969). Algunos aspectos de la nefrosis aviariay de la enfermedad Producida por el agents infecioso de la bolsa de Fabricio en Mexico. Tec. Pecu. Suppl. No.1, 98-104 (Cited by Okoye, 1984).

Gyening, K.O. and corkish, J.D. (1977). Infectious bursal disease in Ghana. Bull Off Int Epizoot 88: 293-298. (Cited by Okoye, 1984).

Heine, H.G., Haritou, M., Failla, P. Fahey, K. and Azad, A. (1991). Sequence analysis and expression of the host protective immunogen VP_2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type I strain. J. Gen. Virol. **72**: 1835-1843.

Helmboldt, C.F. and Garner, E. (1964). Experimentally induced Gumboro disease. Avian Dis. **8**: 561-575.

Herceg, M., Mazija, H. and Kralj, M. (1971). Investigation of Gumboro disease in fowls. I. Data on the first outbreak of the disease in Yogoslavia. Vet. Arh. **41**: 67-71. (Cited by Okoye, 1984).

Hirai, K., Kato, M., Fujiura, A., and Shimakura, S. (1979). Further morphological characterization and structural proteins of infectious bursal disease virus. J. Virol. **32**: 323-328.

Hirai, K. and Shimakura, S. (1974a). Structure of infectious bursal disease virus. J. Virol. **14**: 957-964.

Hirai, K. and Shimakura, S. (1974b). Some properties of precipitating antigens associated with infectious bursal disease virus. Infect. Immun. **10**: 1235-1240.

Hirai, K.,Shimakura, S., Chang, C., Adachi, Y., Kawamoto, E., Taguchi, N., Suzuki, Y., Itakura, C., Funahashi, F., Tsushio, Y. and Hirose, M. (1973). Isolation of infectious bursal disease virus and distribution of precipitating antibodies in chicken sera. Jap. J. Vet. Sci. **35**: 105-113.

Hirai, K., Shimakura, S. and Hirose, M. (1972). Immunodiffusion reaction to avian infectious bursal disease virus. Avian Dis. **16**: 961-964.

Hirose, M. and Hirai, K. (1976). Precipitating antibody against infectious bursal disease virus in egg yolk and serum of chickens. Res. Bull. Fac. Agric. Gifu. Univ. 39: 165-170. (Cited by Kher,1988).

Hitchner, (1970). Infectivity of infectious bursal disease virus for embryonating eggs. Poult. Sci. 49: 511-516.

Hitchner, (1971). Persistance of parental infectious bursal disease antibody and its effect on susceptibility of young chicken. Avian Dis. 15: 894-900.

Hoskins, J.M. (1967). Virological procedures. Butterworth and Co. (publishers), London, pp. 7-54.

Ide, P.R. and Stevenson, R.G. (1973). Infectious bursal disease in New Burswinck. Can. J. Comp. Med. 37: 347-355.

Jackwood, D.J., Frederick, S.B. Kibenge, F.S.B. and Mercado, C.C. (1989). Detection of infectious bursal disease virus by using cloned DNA probes. J. Clin. Micobiol. 27: 2437-2443.

Jackwood, D.J. and Saif, Y.M. (1987). Antigenic diversity of infectious bursal disease virus. Avian Dis. 31: 766-770.

Jackwood, D.J., Saif, Y.M. and Hughes, J.H. (1982). Characteristics and serologic studies of two serotypes of infectious bursal disease virus in turkeys. Avian Dis. 26: 871-878.

Jackwood, D.J., Saif, Y.M. and Hughes, J.H. (1984). Nucleic acid and structural proteins of infectious bursal disease virus isolates belonging to serotype I and II. Avian Dis. 28: 990-1006.

Jackwood, D.J., Saif, Y.M. and Moorhead, P.D. (1985). Immunogenicity and antigenicity of infectious bursal disease virus serotypes I and II in chicken. Avian Dis. 29: 1184-1194.

Jayaramiah, B. and Mallick, B.B. 1974. Identification of infectious bursal disease virus in India. Indian J. Anim. Hlth. 13: 99-103.

Jayaramiah, B. and Mallick, B.B. 1975. Serological survey of infectious bursal disease in India. Indian Vet. J. 52: 673-676.

Jhala, M.K. and Kher, H.N. (1991). Seroprevalence of infectious bursal disease in Gujarat state. Indian Vet. J. 68: 402-405.

Karunakaran, K., Sheriff, F.R., Ganesan, P.I., Kumanan, K. and Gunaseelan (1992). A rare report on infectious bursal disease in ducks. Indian Vet. J. 69: 854-855.

Kaufer, I. and Weiss, E. (1980). Significance of bursa of fabricious as target organ in infectious bursal disease of chickens. Infect.Immun. 27: 364-367.

Kher, H.N. (1988). Isolation antigenic characterization, immunogenic moiety and immunosuppressive factor of infectious bursal disease. Ph.D. thesis, University of Agricultural Sciences, Bangalore.

Kibenge, F.S.B., Dhillon, A.S. and Russel, R.G. (1988). Biochemistry and immunogenicity of infectious bursal disease virus. J. Gen. Virol. 69: 1757-1775.

Kibenge, F.S.B., Jackwood, D.J. and Mercado, C.C. (1990). Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. J. Gen. Virol. 71: 564-577.

Koster, J. and Paulsen, J. (1971). Multiplication of causal agent of infectious bursitis in chick embryo kidney cell cultures. Zentralbl. Vet. Med. 18: 366-372.

Koteeswaran, A. 1987. Study on the pathogenesis of infectious bursal disease virus on chick embryos, chicks and layers and its effect on the immunity against Ranikhet disease. Ph.D. thesis, Tamil Nadu Agricultural University, Coimbatore.

Kouwenhoven, B. (1994). Control of very virulent Gumboro disease in the Netherlands. World Poult., December 15-16.

Kreagar, K. (1994). Infectious bursal disease in egg-type pullets. World Poult., December 19-20.

Kumar, S., Kulschreshtha, R.C. and Kaushik, R.K. (1984). Prevalence of some viral diseases in poultry in Haryana and adjoining states. Haryana Veterinarian 23: 118-120.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680-685.

Lana, D.P., Beisel, C.E. and Silva, R.F. (1992). Genetic mchanism of antigenic variation in infectious bursal disease virus: analysis of naturally occuring variant virus. Virus Genes 6: 247-259.

Landgraf, H., Vielitze, E. and Kirsh, R. (1967). Occurrence of an infectious bursal disease affecting the bursa of Fabricius. Dt tierarztl wschr **74**: 6-10 (Cited by Okoye, 1984).

Lalkrishna (1994). Gumboro disease - present status in India. In. National symposium on advances in veterinary research and their impact on animal health and production, Bareilly India.

Landgraf, H., Vielitze, E. and Kirsch, R. (1967). Untersuchungen Uber das Auftreten einer infektiosenErkrankung mit des Bursa Fabrici (GD). Dt tierarztl wschr **74**: 6-10 (Cited by Okoye, 1984).

Lange, H., Muller, H., Kaufer, I. and Becht, H. (1987). Pathogenic and structural properties of wild type infectious bursal disease virus and virus grown in vitro. Arch. Virol. **92**: 187-196.

Lasher, H.N. and Shane, S.M. (1994). Infectious bursal disease prevention and control in Asia. World Poult., December 26-27.

Lee, L.H., Chang, Y.C. and Shien, J.H. (1992). Adaptation of dot blot hybridization assay for the detection of infectious bursal disease virus RNA. J. Chin. Soc. Vet. Sci. **18**: 59-68.

Lee, L.H. and Lukert, P.D. (1987). The variations of structural protein and nucleic acid of infectious bursal disease virus among different serotypes. J. Chin. Soc. Vet. Sci. **13**: 99-108.

Lensing, H.H. (1968). Gumboro disease. Tijdschr Diergeneesk. **93**: 1452-1460. (Cited by Okoye, 1984).

Louzis, C.,Gillet, J.P., Irgens, K., Jeanin, A. and Picault, J.P. (1979). La Maladie de Guboro apparition Chezelefaisan delevage. Bull. Mens. Soc. Vet. Prat Fr. 63: 785-789.(Cited by Okoye, 1984).

Lucio, B. and Hitchner, S.B. (1979). Infectious bursal disease emulsified vaccine. Effect upon neutralizing antibody levels in the dam and subsequent protection of the progeny. Avian Dis. 23: 466-478.

Lucio, B. and Hitchner (1980). Response of susceptible verses immune chicks to killed, live modified and wild infectious bursal disease virus vaccine. Avian Dis. 24: 1037-1047.

Luker, P.D. (1994). Using live vaccines in the presence of maternal antibody. World Poult., December 12-13.

Lukert, P.D., and Davis, R.B. (1974). Infectious bursal disease virus: growth and characterization in cell cultures. Avian Dis. 18: 243-250.

Lukert, P.D. and Hitchner, S.B. (1984). Infectious bursal disease in "Diseases of Poultry" edited by Hostad, M.S., Barnes, H.J., Calnek, B.W. Reid, W.M. and Yoder, H.W. Jr. 8th Ed. Iowa State University Press, Ames, pp. 566-576.

Lukert, P.D. and Mazariegos, L.A. (1985). Virulence and immunosuppressive potential of intermediate vaccine strains of IBD. J. Am. Vet. Med. Assoc. 187: 306.

Lukert, P.D. and Saif, Y.M. (1991). Infectious bursal disease. In. "Diseases of Poultry", edited by Calnek, B.W., Barnes, H.J., Beard, C.W., Reid, W.M. and Yoder, H.W. Jr. 9th Edn, Wolfe Publishing Ltd., England. pp. 648-663.

Lyutskanov, D., Nikolov, N., Lipov, I. and Stamenov, M. (1978). Clinical and epizootiological studies on Gumboro disease. Vet. Medi. Nauki Sofia. **15**: 69-75 (Cited by Okoye, 1984).

Maire, C., Renault, L., Alamagny, A., Dreuille, M.de and Albouy, R. (1969). Existence en France de la maladi de Gumboro. Recl. Med. Vet. **145**: 75-84. (Vet. Bull. **39**:3927).

Mallinson, E.T., Snyder, D.B., Marquardt, W.W., Cohen, E., Savage, P.K., allen, D.C. and Yancey, F.S. (1985). Presumptive diagnosis of subclinical infections utilysing ELISA against multiple antigens. Poult. Sci. **64**: 1661-1669.

Mandelli, G., Lodetti, E., Rinaldi, A. and Cervio, G. (1972). Charactristiche colturali citologiche ed ultra-microscopiche di un cepoo di virus della malattia di Gumboro (IBA-1/pr). Fol. Vet. Lat. **21**: 399-425 (Cited by Okoya, 1984).

Mandelli, G., Rinaldi, A., Cessi, D., Cervio,G., Pascucci, S. and Vleni, A. (1969). Experimental infection of day old chicks with Gumboro disease virus. Atti. Soc. ital Soci. Vet. **23**: 1070-1074 (Vet. Bull. **41**: 193).

Marquardt, W.W., Jhonson, R.B., Odenwald, W.F. and Schlottaber(1980). An indirect ELISA for measuring antibodies in chickens infected with infectious bursal disease virus. Avian Dis. **24**: 375-385.

McFerren, J.B., McNulty, M.S., Mc Killop, E.R., Cannon, T.J., Mc Cracken, R.M., Collins, D.S. and Allen, G.M. (1980) Isolation and serological studies with infectious bursal disease virus isolates belonging to serotype I and II. Avian Pathol. **9**: 395-404.

McNulty, M.S., Allan, G.M. and McFerren, J.B. (1979). Isolation of infectious bursal disease viruse from turkeys. Avian Pathol. **8**: 205-212.

Mekkes, D.R. and deWit, J.J. (1994). Diagnostic tools in detection of variant and virulent strains and antibody. World Poult., December 10-11.

Meroz, M. (1966). An epidemiological survey of Gumboro disease. Refuah. Vet. **23**: 235-237. (Cited by Okoye, 1984).

Meulmans, G. and Halen, P. (1977). The immunodeppressive effect of infectius bursal agent on vaccination against Newcastle disease. Res. Vet. Sci. **22**: 222-224.

Mohanty, G.C. and Kradjian, Y.S.,Aziz, S.S. and Al-Wahab, N.M.A. (1981). Occurrence of infectious bursal disease of chicken in Iraq. Indian J. Poult. Sci. **16**: 251-258.

Mohanty, G.C., Pandey, A.P. and Rajya, B.S. (1971). Infectious bursal disease in chickens. Curr. Sci. **40**: 181-184.

Mohanty, G.C. and Rao, A.T. (1984). Studies on natural and experimental infection with infectious bursal disease in Orissa. Indian J. Poult. Sci. **19**: 210-213.

Muller, H. and Becht, H. (1982). Biosynthesis of virus-specific proteins in cells infected with infectious bursal diseases virus and their significance as structural elements for infectious virus and incomplete particles. J. Virol. **44**: 384-392.

Muller, H., Schnitzler, D., Bernstein, F., Becht, H., Cornelissen, D. and Lutticken, D.H. (1992). Infectious bursal disease of poultry: antigenic structure of the virus and control. Vet. Microbiol. 33: 175-183.

Muller, H., Scholtisser, C. and Becht, H. (1979). The Genome of infectious bursal disease virus consists of two segments of double stranded RNA. J. Virol. 31: 584-589.

Nachimuthu, K., Dhinakar Raj, G., Thangavelu, A., Koteeswaran, A., Palaniswamy, K.S. and Venugoplan, A.T. (1993). Seroprevalence of IBD in Tamil Nadu. Indian Vet. J. 70: 100-103.

Nakano, M., Postugal, M.A.S.C., Saliba, A.M., Nobre, D. and Narimatsu, M.N. (1972). Ocorrencia de doenca de Gumboro no Brazil. Diagnostico anatomo. Pathologico. Biologico 38: 60-61 (Cited by Okoye, 1984).

Naqi, S.A., Marquez, B. and Sahin, N. (1983). Maternal antibody and its effect on infectious bursal disease immunization. Avian Dis. 27: 623-631.

Naqi, S.A., Millan, D.L. and Grumbles, L.C. (1980). An evaluation of three commercially available infectious bursal disease vaccines. Avian Dis. 24: 233-240.

Nick, H., Cursiefen, D. and Becht, H. (1976). Structural and growth characteristics of infectious bursal disease virus. J. Virol. 18: 227-234.

Okoye, J.O.A. (1984). Infectious bursal disease of chickens. Vet. Bull. 54: 425-436.

Okoye, J.O.A. and Uzoukwu, M. (1981). An outbreak of infectious bursal disease among chickens beween 16 and 20 week old. Avian Dis. 25: 1034-1038.

Onunkwo, O. (1978). Problems of Gumboro disease of chickens in Nigeria. J. Nig. Poult. Sci. Assoc. 5: 33-35.

Oppling, V., Muller, H. and Becht, H. (1991). Heterogenecity of the antigenic site responsible for the induction of neutralizing antibodies in infectious bursal disease virus. Arch. Virol. 119: 211-223.

Panigrahy, B., Misra, L.K. and Adams, L.G. (1982). Humoral and cell mediated immune response in chickens with infectious bursal disease. Vet. Microbiol. 7: 383-387.

Panisup, A.S., Verma, K.C., Kataria, J.M., Singh, S.D. and Mohanty, G.C. (1987). Infectious bursal disease in chicken: Disease status precipitated condition and experimental studies. Indian J. Poult. Sci. 22: 245-250.

Perelman, B. and Heller, E.D. (1981). Preliminary serological survey for infectious bursal disease in turkeys in Israel. Refuah Vet. 38: 12-16.

Petek, M., D'April, P.N. and Cancellotti (1973). Biological and physicochemical properties of the infectious bursal disease virus. Avian Pathol. 2: 135-152.

Petek, M. and Mandelli, G. (1968). Biological properties of reovirus isolated in an outbreak of Gumboro disease. Atti. Soc. ital. Sci. Vet. 22: 875-879 (Vet. Bull. 39: 4246).

Philips, Jr. W.E. (1981). Comparison of precipitatingantibodies and virus neutralizing antibodies to infectious bursal disease virus. Avian Dis. 25: 1093-1097.

Pradhan, H.K., Mohanty, G.C., Verma, K.C. and Panda, B.K. (1983). Evidence of some chicken viral infections in Japanese quails based on serological studies. Indian J. Poult. Sci. **18**: 81-84.

Provost, A., Borredon, C. and Bocquet, P. (1972). Deuxmaladies aviaires nouvelles au Tchad: la laryngotracheite infectieus et la Maladie de Gumboro. Rev. Elev. Med. Vet. **25**: 347-385. (Cited by Okoye, 1984).

Purushothaman, V., Panneerselvam, S., Venugopal, K. and Thangaraj, T.M. (1988). A report on the outbreak of infectious bursal disease in chicken. Cheiron **17**: 229-231.

Rao, A.T., Nayak, B.C., Das, B.C. and Parhi, N.K. (1979). concurrent infection of infectious bursal disease and Newcastle disease in Orissa - A pathological study. Indian J. Poult. Sci. **14**: Suppl. No.1, 6-7.

Rao, V.N. and Rao, K.N.P. (1992). Immunosuppression to Ranikhet disease due to infectious bursal disease virus in chicken. Indian Vet. J. **69**: 686-689.

Rao, S.U.M., Sambamurti, B. and Mallick, B.B. (1978). Dwarfing and mortality of chicken embryos caused by indigenous isolates of infectious bursal disease virus. Indian J. Anim. Sci. **48**: 111-114.

Ratanasethakul (1983). Infectious bursal disease in broiler chickens. Thai. J. Vet. Med. **13**: 65-78. (Quoted by Kher, 1988).

Ray, D.K. and Sarkar, P.K. (1984). Seroprevalence of infectious bursal disease in poultry around Calcutta. Indian J. Poult. Sci. **19**: 224-227.

Riggenbach, C. (1967). Apparition de la maladie de Gumboro en suisse. Schweizer Arch. Tierheilk. **109**: 398-400. (Vet. Bull. **38**: 1576).

Rinaldi, A., Cervio, G. and Mandelli, G. (1965). Comparsadi una nuova form a morbosa dei polli negli allevamenti avicoli della Lombardia Verosimilmenta identificabile conla cosidetta malattia di Gumboro. Sel. Vet. **6**: 207-209.

Rosales, A.G. (1994). Control programmes and assessment of immunity. World Poult., December 21-23.

Rosenberger, J.K. and Gelb (1978). Response to several avian respiratory viruses as affected by infectious bursal disease virus. Avian Dis. **22**: 95-105.

Rosenberger, J.K., Klopp, S., Fackroode, R.J. and Krauss, W.C. (1975). The role of infectious bursal agent and several avian adenoviruses in the haemorrhagic aplastic anaemia syndrome and gangrenous dermatitis. Avian Dis. **19**: 717-729.

Sagna, F. (1977). Une nouvelle affection aviaire an Senegal; la maladie de Gumboro. Bull. Off. Int. Epizoot **88**: 281-290. (Cited by Okoye, 1984).

Saif, Y.M. (1991). Immunosuppression induced by infectious bursal disease virus. Vet. Immunol. Immunopathol. **30**: 45-50.

Saif, Y.M. (1994). Laboratory versus live bird testing. World Poult., December 9-11.

Saijo, K., Higashihasa, M., Fujisaki, Y. and Matumoto, M. (1990). Isolation and characterization of attenuated plaque variants of infectious bursal disease virus. Vet. Microbiol. 22: 171-178.

Sharma, R.N., Benko, L. and D'Cruz, S. (1977). Preliminary observations on infectious bursal disease in Zambia.Vet. Rec. 101: 153.

Sharma, J.M., Dupuy, J.M. and Lamontagne, L. (1989). Immuno-suppression by avian infectious bursal disease virus and mouse hepatitis virus. In "Induced immuno-suppression". Plenum Pres. New York, NY.pp. 201-216.

Schneider, J. and Hass, K. (1969). Untersuchugen Zur Atiologic der infektiosen Bursitis (Gumboro disease) bei Junghennen Und Kuken. Berl. Munch. tierarztl. Wschr. 82: 252-255 (Cited by Faragher, 1972).

Shimizu, F., Hasegawa, I. and Tamizawa, M. (1971). Isolation of the infectious bursal agent from field chickens in Japan. Bull. Natn. Inst. Anim. Hlth. Tokyo. No.63, pp. 1-5 (Cited by Faragher, 1972).

Singh, K.C.P. and Dhawedkar, R.G. (1992). Growth of different strains of infectious bursal disease virus in cell culture. Indian J. Virol. 8: 15-17.

Singh, K.C.P. and Dhawedkar, R.G. (1993). Serological characterization of local strains of infectious bursal disease virus and their antigenic relation with a vaccine virus. Indian J. Virol. 9: 120-124.

Singh, K.C.P. and Dhawedkar, R.C. (1994). Effect of infectious bursal disease virus on cell mediated immune response of chickens. Indian J. Virol. 10: 90-96.

Singh, K.C.P., Verma, S.K., Prasad, C.B. (1994). Occurrence of infectious bursal disease in chickens, isolation and clinico-pathology. Indian J. Virol. **10**: 83-89.

Skeeles, J.K. and Lukert, P.D. (1980). Studies with an attenuated cell-culture adapted infectious bursal disease virus. Replication sites and persistence of the virus in SPF chickens. Avian Dis. **24**: 43-47.

Skeeles, J.K., Lukert, P.D.,Fletcher, D.J. and Leonard, J.D. (1979). Immunization studies with a cell-culture adopted infectious bursal disease virus. Avian Dis. **23**: 456-465.

Snyder, D.B., Marquardt, W.W., Mallinson, E.T., Cohen, K.E., Savage, P.K. and Allen, D.C. 1986. Rapid serological profiling by ELISA. IV. Association infectious bursal disease serology with broiler flock performance. Avian Dis. **30**: 139-148.

Snyden, D.B., Yancey, F.S. and Savage, P.K. (1992). A monoclonal antibody based AGPT for antigenic assessment of infectious bursal disease virus. Avian Pathol. **21**: 153-157.

Solona, W., Giambrone, J.J. and Panangala, V.S. (1985). Comparison of a kinetic based enzyme-linked immunosorbant assay (KELISA) and virus neutralization test for infectious bursal disease virus. I. quantitation of antibody in White Leghorn hens. Avian Dis. **29**: 662-671.

Somvanshi, R., Mohanty, G.C., Kataria, J.M. and Verma, K.C. (1985). Detection of infectious bursal disease antibodies and antigen by counter immunoelectrophoresis. Indian J. Poult. Sci. **20**: 66-67.

Sudhakaran, R., Mohan Reddy, A.R., Padmavathi, G., Shakila, S. and Kalpana, G. (1993). A report on an epidemic of infectious bursal disease in poultry in Chittoor district of Andhra Pradesh. Poult. Adv. **26**: 29-31.

Sulochana, S. and Lalithakunjamma, C.R. (1991). Infectious bursal disease in Kerala. Cheiron. **20**: 181-183.

Sureshkumar, S., Mishra, R.C., Kulshreshtha, R.C. and Kulsik (1984). An outbreak of infectious bursal disease in 25-26 week old layers. Indian J. Comp. Microbiol. Infect. Dis. **15**: 123-125.

Survanshe, B.D. (1990). Watch out for Gumboro. Poult. Misset. June-July, 37-38.

Talwar, G.P. (1983). Immunoelectrophoresis. In "Handbook of Practical Immunology". Vikas Publishing House, New Delhi. pp. 122-133.

Thornton, D.H. and Pattison, M. (1975). Comparison of vaccines against infectious bursal disease. J. Comp. Pathol. **85**: 597-610.

Ture, O., Tsai, H.J. and Saif, Y.M. (1993). Studies on antigenic relatedness of classic and variant strains of infectious bursal disease virus. Avian Dis. **37**: 647-652.

Valsala, K.V., Nair, M.K., Koshy, V. and Rajan, A. (1988). Prevalence of infectious bursal disease in Kerala. Kerala J. Vet. Sci. **19**: 60-64.

Van den berg, T.P., Ganze, M. and Meulmans, G. (1991). Acute infectious bursal disease in poultry, isolation and characterisation of highly virulent strain. Avian Pathol. **20**: 133-143.

Vasicek, L. (1979). Untersuchugen zum Vorkommen der Infectiosen Bursitis (Gumboro Krankheit). Wein tierarztl muschr 66: 116-123. (Cited by Okoye, 1984).

Verma, K.C., Panisup, A.S. and Mohanty, G.C. (1990). Infectious bursal disease in chicken. Indian Veterinary Research Institute, Izatnagar. Res. Pub. No.1.

Verma, K.C., Panisup, A.S., Mohanty, G.C. and Reddy, B.D. (1981). Infectious bursal disease and associated conditions in poultry flocks of Andhra Pradesh. Indian J. Poult. Sci. 16: 385-392.

Verma, N.D., Sharma, D. and Ghosh, S.S. (1989). Serological screening against common infections of poultry in Tripura state. Indian J. Anim. Sci. 59: 362-364.

Vielitz, E. and Landgraf, H. (1976). Comparative tests on safety and potency of infectious bursal agent vaccines. Dev. Biol. Stand. 33: 332-339 (Vet. Bull. 48: 5410).

Vijayan, N., Valsala, K.V., Manomohan, C.B., Koshy, V. and Nair, M.K. (1989). Histopathological observations on the organs in infectious bursal disease of chicken. Kerala J. Vet. Sci. 20: 139-143.

Vijayapraveen, K., Seshagiri Rao, A. and Satyanarayanachetty, M. (1995). Isolation, identification and characterization of infectious bursal disease virus in Andhra Pradesh. Indian Vet. J. 72: 5-9.

Wangner, K. and Koster, J. (1968). Serological studies on infectious bursitis in chicks (Gumboro disease). Berl Munch tierarztl Wschr 81: 464-466 (Vet. Bull. 39: 2651).

Weisman, J. and Hitchner, S.B. (1978). Virus neutralization versus agar gel precipitin tests for detecting serological response to infectious bursal disease virus. Avian Dis. 22: 598-603.

Winterfield, R.W. (1969). Immunity response to infectious bursal agent. Avian Dis. 13: 548-557.

Winterfield, R.W., Dhillon, A.S., Thacker, H.C. and Alby, L.J. (1980). Immune response of White Leghorn chicks from vaccination with different strains of infectious bursal disease virus and in the presence of maternal antibodies. Avian Dis. 24: 179-188.

Winterfield, R.W. and Fadly, A. (1971). Infectious bursal disease immunity. Proc. 19th World Vet. Cong., Mexico 20: 502-504 (Vet. Bull. 42: 1865).

Winterfield, R.W. and Thacker, H.L. (1978). Immune response and pathogenicity of different strains of infectious bursal disease virus applied as vaccines. Avian Dis. 22: 721-731.

Wood, G.W., Muskett, J.C., Hebert, C.N. and Thornton, D.H. (1979). Standardization of the quantitative agar gel precipitation test for antibodies to infectious bursal disease virus. J. Biol. Std. 12: 311-314.

Wood, G.W., Muskett, J.C. and Thornton, D.H. (1981). The interaction of live vaccine and maternal antibody in protection against infectious bursal disease. Avian Pathol. 10: 365-373.

Wyeth, P.J. (1975). Effect of infectious bursal disease on the response of chickens to S. Typhimurium and E. coli. infections. Vet. Rec. 96: 238-243.

Wyeth, P.J. (1980). Passively transferred immunity to infectious bursal disease following live vaccination of parent chickens by two different routes. Vet. Rec. 106: 289-290.

Wyeth, P.J. and Cullen, G.A. (1976). Maternally derived antibody on susceptibility of chicks to infectious bursal disease. Avian Pathol. 5: 253.

Wyeth, P.J. and Cullen, G.A. (1978a). Transmission of immunity from inactivated infectious bursal disease oil-emulsionvaccinated parent in their chicks. Vet. Rec. 102: 368.

Wyeth, P.J. and Cullen, G.A. (1978b). Susceptibility of chicks to infectious bursal agent following vaccination of their parents with live infectious bursal disease vaccine. Vet. Rec. 103: 281-282.

Yamaguchi, S., Imada, I. and Kawamura, H. (1981). Growth and infectivity titration of virulent infectious bursal disease virus in established cell lines from lymphoid leucosis. Avian Dis. 25: 927-935.

Zhou, J., Lin, F.Z., Chen, I.Y. and Wang, S.J. (1982). Isolation of the infectious bursal disease agent of chickens in the Beijing area. Chin. J. Vet. Med. 8: 25-26. (Vet. Bull. 53: 5307).

STRUCTURAL ANALYSES OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM CLINICAL CASES IN VACCINATED AND UNVACCINATED BIRDS

By

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ABSTRACT OF A THESIS

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ABSTRACT

Field cases with history suggestive of infectious bursal disease (IBD) were screened for confirmation by Agar gel diffusion test (AGDT), using reference antigen and antisera received from Madras Veterinary College. From the positive cases, 4 isolates, two each from unvaccinated (PKD, EKM) and vaccinated (THR, KAN) flocks and an avirulent vaccine strain (VAC) were used for structural analyses and antigenic relationship studies.

The percentage of mortality of the embryos infected by these strains ranged between 50-100 per cent, during the third and fifth day of inoculation, in the fourth passage. The lesions produced were cutaneous haemorrhages all over the body, congestion and thickening of CAM. Enlarged bursa and typical yellowish green discolouration of liver with brown patches were also noticed. All the five isolates were propagated in chicken embryo fibroblast culture, in which cytopathic changes characterised by rounding and subsequent detachment was seen from the third passage onwards.

The chicks infected with field isolates revealed mildclinical symptoms. The lesions noticed after sacrificing them on the third day were moderately swollen gelatinous bursa with slight haemorrhage in some of them. Chicks that

received vaccine strain revealed only mild lesion. The viral strains by SDS-PAGE revealed that all the field isolates contained nine identical polypeptides with molecular weights of 86 KD (VP_2), 77 KD (VP_4), 73 KD (VP_5), 62 KD (VP_6), 52 KD (VP_7), 47 KD (VP_8), 39 KD (VP_{10}), 36 KD (VP_{11}) and 32 KD (VP_{12}). The vaccine strain resolved 11 peptides of whichthree, namely VP_1 (93 KD), VP_3 (80 KD) and VP_9 (43 KD), were absent in the field isolates, but it lacked VP_7 (52 KD). Mild difference in the molecular weights of VP_6 and VP_{12} were also noticed between the field isolates and vaccine strain.

Nucleic acid analyses in agarose gel showed two bands for all the five isolates without any difference in their migration pattern.

Antigenic relationship of the IBDV isolates was studied by AGDT, CIE and IE. All the four isolates produced only one precipitation line against the antiserum and this precipitation line was identical to the one produced by the vaccine strain.

From the observations made, the possible reasons for breakdown of immunity and a schedule of vaccination to overcome this situation have been discussed.

Appendix

Enquiry of farmers about the history of the birds that are brought for postmortem examination with the suspicion of infectious bursal disease

1. Name of the owner :

2. Address of the farm :

3. Age of the bird :

4. Type of the bird broiler/ layer :

5. Size of the farm (Number of birds) :

6. Number of birds died :

7. Vaccination done : Yes/No

8. Age at vaccination done :

9. Name of the vaccine (company) if known :

10. Name of the hatchery from which birds are received :

Place:

Date :

Please despatch to:

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Mannuthy, Thrissur-680 651